TOTAL PLASMA HOMOCYSTEINE, VITAMINS, ABDOMINAL AORTIC ANEURYSM AND PERIPHERAL VASCULAR DISEASE

by

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ABSTRACT

Title

Total Plasma Homocysteine, Aortic Aneurysm, Peripheral Vascular Disease and Vitamins.

Introduction

Hyperhomocysteinemia (>15μmol/L) is a recognised independent risk factor in the genesis of vascular diseases. There are few data on the relationship between homocysteine and abdominal aortic aneurysm (AAA), peripheral vascular disease (PVD), and the effect of vitamin B_{12} and folic acid blood levels on plasma homocysteine concentration. This study was aimed to examine this possible relationship.

Method

Ethical approval was obtained for this case-control study. Fasting homocysteine blood levels were analysed using a fluorescence polarisation immunoassay technique. Serum vitamin B_{12} and folic acid was analysed using chemiluminescence detection technique. Serum was separated within one hour of blood collection from antecubital venous puncture into an EDTA primed tube, from 38 patients with AAA, 36 patients with PVD and 36 control subjects for analysis of homocysteine, vitamin B_{12} and folic acid.

Results

Twenty-six (68%) patients with AAA and 26 (72%) patients with PVD patients had elevated levels of plasma homocysteine (>15μmol/L) compared with 2 (6%) in the

case controls. The mean homocysteine levels in the AAA, PVD and control groups were 19.4 ± SE 1.1 (95% CI 17.17 – 21.65), 18.4 ± SE 0.47 (95% CI 16.32 – 20.35) and 10.9 ± SE 1.0 μmols/L (95% CI 9.95 – 11.88) respectively. The difference in mean levels of homocysteine in the AAA vs the control and the PVD vs control was statistically significant (P< 0.001). Mean vitamin B_{12} in the AAA, PVD and control groups was 332.11 ± SE 16.44 pg/L , 320.47 ± SE 16.81 pg/L and 414.33 ± SE 19.72 pg/L respectively. The difference in the mean levels of serum vitamin B_{12} in the AAA vs the control group and the PVD vs the control groups were statistically significant (P < 0.004). Mean serum folic acid for AAA, PVD and control was 8.02 ± SE 0.71 ηgm /L , 7.84 ± SE 0.81 ηgm /L and 9.80 ± SE 0.69 ηgm /L respectively (P> 0.05). However, there was an inverse relationship between plasma homocysteine and B_{12} (r = -0.420, P = 0.000) and, plasma homocysteine and folic acid (r = -0.326, P = 0.001).

Conclusion

This study shows significantly higher levels of plasma homocysteine in patients with AAA and PVD in comparison to control. Use of supplemental vitamins that can lower plasma homocysteine, may modify vascular disease progression. Clinical trials in this direction are warranted.

CERTIFICATE OF RESEARCH

This is to certify that the work described in this thesis has been done by me under the supervision of Professor Bruce Davies at the School of Applied Sciences, University of Glamorgan and Mr. M H Lewis at the Royal Glamorgan Hospital and that no part of it has been produced and or presented for any other research work.

Signed: .....

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Signed: .....

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DEDICATION

Dedicated to my parents, family and friends without whose help and support this thesis would not have been completed

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The heights by great men reached and kept

Were not attained by sudden flight

But they while their companions slept

Were toiling upwards in the night

H. Longfellow

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Glossary of Nomenclature and Abbreviations

AAA- abdominal aortic aneurysm

ABPI- ankle brachial pressure index

ALI- acute limb ischemia

AP- anterio-posterior diameter

B_6- pyridoxine

B_{12}- cobalamin

CAD- coronary artery disease

CC- cytosine cytosine

CI- confidence interval

CLI- chronic limb ischemia

COAD- chronic obstructive disease

CTScan- computerised tomography scan

CVD- cerebrovascular disease

DNA-deoxyribonucleic acid

ECG- electrocardiogram

EDTA- ethylene diamine tetra acetic acid

ESR- erythrocyte sedimentation rate

FBC- full blood count

FPIA- fluorescent polarisation immunoassay

GAG- glycosamine glycans

H_0- null hypothesis

H_2O_2- hydrogen peroxide

Hcy- plasma homocysteine

HcyT- homocysteine thiolactone

HMMT- homocysteine methionine methyl transferase

HUVE- human umbilical vein endothelium

LDL- low density lipoprotein

MRI- magnetic resonance imaging

MTHFR- methylene tetrahydrofolate reductase

ns- not significant

'P'- probability

PAPS- phosphoadenosine phosphosulphate

PGI_2- prostaglandin I_2

PTFE- polytetrafluoroethylene

PVD- peripheral vascular disease

RPM- revolutions per minute

SAM- s-adenosyl methionine

SD- standard deviation

SE- standard error

SPSS- statistical package for social science

TC- thymidine cytosine

TF- tissue factor

THcy- total plasma homocysteine

TT- thymidine thymidine

UEC- urea electrolyte creatinine

USS- ultrasound scan

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Research communication associated with thesis

Publications

1. **Abdominal aortic aneurysms and its correlation to plasma homocysteine and vitamins**
   Ali A.Warsi, G. Morris-Stiff, D. Hullin, B. Davies, M.H. Lewis.
   In press in the *European Journal of Vascular and Endovascular surgery 2003.*

2. **Correlation between plasma homocysteine, aortic aneurysms and vitamins**
   Ali A.Warsi, D. Hullin, B. Davies, M.H. Lewis.
   Abstract published in the *European Surgical Research, Vol. 34 (Supl 1), May 2002, 36.*

Presentations

3. **Hyperhomocysteinaemia, abdominal aortic aneurysm and vitamins**
   Ali A.Warsi, D. Hullin, B. Davies, M.H. Lewis.
   Accepted for presentation at the 37th Congress of the European Society for Surgical Research (ESSR), Hungary, May 2002.

4. **Hyperhomocysteinaemia and its correlation with abdominal aortic aneurysm**
   Ali A. Warsi, B. Davies, MH Lewis.
   Presented at the Annual Regional Research Day meeting, East Glamorgan Hospital, Oct. 1999, Pontypridd, S. Wales.

Chapter 1

General overview

1.1 Introduction

For more than 20 years, raised plasma total homocysteine (THcy) has been associated with risk of atherosclerotic and thrombotic event. In fact, it is now nearly 40 years since attention was first drawn to the possible relationship of raised plasma homocysteine to premature vascular diseases in patients who suffered from homocysteinuria. Recently, evidence has mounted to suggest that the association may be causal. This association is independent of other known risk factors such as hypertension, hyperlipidemia, hypercholesterolemia, diabetes and smoking. The association is fairly consistent across many studies, is strong and dose-related, and is biologically plausible. Homocysteine is a sulphur containing amino acid, elevated plasma levels of which are referred to as hyperhomocysteinemia. It is an intermediary product in methionine metabolism. The transfer of methyl group from methionine is an important step in the metabolism of nucleic acids, fats, and high-energy bonds. When methionine donates its methyl group, homocysteine is formed. The majority of homocysteine is recycled in a transmethylation reaction involving vitamin B_{12} and folic acid while a smaller amount is metabolised by transulphuration involving vitamin B_6. Hence the vitamin intake and serum vitamin levels play an important part in the regulation of plasma homocysteine levels. Whereas the stenotic lesions such as coronary artery disease (CAD), cerebrovascular disease (CVD) and peripheral vascular disease (PVD) and its correlation to plasma homocysteine has received much of the attention in scientific literature, there is very little data that on the relationship between hyperhomocysteinemia and arterial aneurysm. The pathology of the aneurysmal disorder is largely similar to stenotic lesion with the basic pathology being atherosclerosis. In addition, there are other factors such as serine elastase and matrix metalloproteinases that have an influence in the genesis of aneurysmal disorder

suggesting that there may be a genetic, biochemical and nutritional basis of elevated plasma homocysteine.

Annual mortality from peripheral vascular disease, and abdominal aortic aneurysm (AAA) in the U.K. is estimated to be more than 2,500 and about 6,000 per annum respectively (Office of Population Censuses and Surveys. 1995). Thus AAA and PVD are common vascular diseases. To prevent their development and arrest their progression by identification and reduction of risks such as lowering of plasma homocysteine would contribute to health care improvement. The relationship between AAA and elevated plasma homocysteine, folic acid, and vitamin B_{12} remains to be established, which this study intends to address.

The aims and objectives of the study were to address the relationship between plasma homocysteine, aneurysms, peripheral vascular disease, and vitamins, by means of case-control study.

In study 1, patients with abdominal aortic aneurysm (AAA) and age matched controls without abdominal aortic aneurysm were selected. Though there can be aneurysms anywhere in the arterial tree in the human body, the researcher selected abdominal aortic aneurysm because of its clinical importance and significance, the ease of an objective assessment of its size and the availability of patients with this disorder in the vascular surgical unit at the Royal Glamorgan Hospital. The study measured folic acid, vitamin B_{12} since several studies have shown their inverse relationship to plasma homocysteine in the genesis of atherosclerotic vascular disorder.

In study 2, the researcher aimed to measure the plasma homocysteine and its relationship to peripheral vascular disease (PVD) in comparison to controls who were objectively assessed not to suffer from peripheral vascular disease. The aim was to examine the possible relationship between plasma homocysteine and peripheral vascular disease in the population in Wales, since there has been no study that has examined such an association. If the relationship demonstrated in study 2 was similar to those reported in the literature, this would confirm that the effect of elevated plasma homocysteine on vascular disorder is no different in the Welsh population than those shown in studies around the world. This result would act as an additional control or benchmark for comparison to the results of study 1. Folic acid and vitamin B_{12} were also measured, since several studies have shown their inverse relationship to plasma homocysteine in the genesis of atherosclerotic vascular disorder.

Chapter 2

Review of literature

2.0 Homocysteine

2.1 Introduction

Homocysteine is a sulphur containing amino acid and elevated levels of homocysteine are commonly referred to as hyperhomocysteinemia. The normal range of fasting plasma homocysteine is 5-15μmol/L (Still and McDowell, 1998; Refsum et al., 1997). The terms mild, moderate and severe hyperhomocysteinemia refer to elevated plasma homocysteine <30μmol/L, 30 - 100μmol/L and >100μmol/L respectively. Even minor elevations of homocysteine have been implicated in the pathogenesis of vascular disorders (Boushey et al., 1995). In exercising its effect on vascular diseases, elevated levels of homocysteine have been shown to be a major independent risk factor (Graham et al., 1997; Boushey et al., 1995; Clarke et al., 1991). The effect on the arteries can cause atherosclerotic stenotic lesions involving the cerebrovascular, cardiovascular and peripheral vascular vessels (Boers et al., 1985; Brattstrom et al., 1984; Wilcken and Wilcken, 1976). Their respective clinical manifestation results in stroke, myocardial infarction or angina and intermittent claudication or rest pain. However, if stenosis or narrowing of the arteries represents one end of the spectrum of pathological effects of raised homocysteine, aneurysm formation or dilatation of the arteries may represent the other end. Whereas, stenotic lesions have received much attention in the scientific literature, there has been only isolated reported cases of raised homocysteine associated with aneurysm formation (Almgren et al., 1978; Colwell et al., 1991; Mohan et al., 1997) and one prospective study of raised homocysteine and abdominal aortic aneurysm (AAA) (Brunelli et al., 2000). Almgren et al. (1978) reported the first case of abdominal aortic aneurysm in a 35 year-old-man with homocysteinuria. This patient also had symptoms of peripheral vascular disease. The diagnosis of AAA was confirmed on aortography and AAA was

repaired surgically. Homocysteinuria was treated with pyridoxine and subsequent test for homocysteinuria was negative. Colwell et al. (1991) reported multiple aneurysms and hyperhomocysteinemia in a 64-year-old male. On clinical examination he had no known risk factors for aneurysm but had mild hypertension. All the laboratory tests were also normal for risk factors. Angiogram revealed multiple aneurysms- aortic, iliac, femoral and popliteal. Plasma homocysteine level was 39 μmol/L. Pathology showed moderate intimal thickening, fibroelastosis, and extensive fragmentation and degeneration of the tunica media. Moreover, enzyme cystathione β synthetase was markedly reduced, thus explaining the basis for elevated plasma homocysteine. The patient underwent elective surgical repair of the aneurysms and made an uneventful recovery. Mohan et al. (1997) reported an association of hyperhomocysteinemia with an 11 cm external iliac aneurysm in a 66-year-old man. The patient had otherwise normal peripheral pulses and no evidence of other aneurysmal disease. Haematological investigation revealed that the patient had macrocytic anaemia despite normal levels of vitamin B_{12} and folic acid, possibly because the patient was an alcoholic. The plasma homocysteine level was 56 μmol/L. Pathology showed a thickened vascular wall with medial fibrosis, fragmentation of the elastic tissue network due to hyaline and mucinoid degeneration and cystic medial change. Bacterial and fungal cultures were negative. Genetic testing revealed that the patient was heterozygous positive for the MTHFR C667T transition. The patient underwent surgical repair of the aneurysm and made an uneventful recovery and was discharged home. It is worth noting that this patient had no known risk factors for aneurysms. These case reports were the only evidence to suggest elevated plasma homocysteine levels in the presence of abdominal aortic aneurysms, until Brunelli et al. (2000) demonstrated that patients with AAA had significantly higher levels of homocysteine in comparison to control (see section on pathogenesis – aneurysm). AAA is by far the

most important of the aneurysms due to the very high mortality exceeding 80% associated with its rupture (Basnyat et al., 1999). The relationship of raised plasma homocysteine and abdominal aortic aneurysm therefore remains largely unexplored.

2.2 History and Background

Gibson (1964) and Shimke (1965) first described the correlation between hyperhomocysteinemia and vascular diseases in the early sixties on patients with the inherited disorder of homocysteinuria. It was the observation that patients suffering from homocysteinuria, an autosomal recessive metabolic disorder of raised plasma homocysteine, suffered from various vascular disorders that drew attention to the causal relationship with homocysteine. McCully (1969) suggested that raised plasma homocysteine is involved in the pathogenesis of atherosclerosis. Indeed, homozygous (when the 2 alleles, one from the maternal derived chromosome and the other from paternal derived chromosome, at a given locus or point along a chromosome are identical) patients for a recessive metabolic disorder affecting the plasma concentration of homocysteine present frequent life-threatening arterial and venous thrombo-embolic events. Harker (1976) conducted an experiment on baboons by artificially inducing homocysteinemia. A solution of homocysteine was infused continuously by femoral AV shunt throughout a 3-month period. 0.3 gm of homocysteine per kg body weight was infused per day (3 gm dissolved in 100 mls of 0.15 M NaCl solution at the pH 7.4). The homocysteine infusion was given at the rate of about 4 ml/hr, which produced a plasma concentration of 0.1-0.2 mM. Silver nitrate staining, light and electron microscopic examination of section of the thoracic and abdominal aorta, iliac and femoral arteries revealed endothelial vascular injury and atherosclerosis. Further studies demonstrated that elevated levels of homocysteine

was a risk factor in the causation of coronary artery (Wilcken and Wilcken, 1976), cerebrovascular (Brattstrom et al., 1984) and peripheral vascular diseases (Boers et al., 1985). Subsequently, it was demonstrated that the effect of hyperhomocysteinemia was independent of other risk factors such as age, gender, lipids, lipoproteins, cholesterol, hypertension, diabetes and smoking. (Graham et al., 1997; Boushey et al., 1995; Clarke et al., 1991).

With regards to the aneurysm formation and homocysteine, the association was first described by Almgren et al. (1978) in a case report of a 35-year old man with ruptured AAA. Colwell et al. (1991) reported a case of multiple aneurysms and hyper homocysteinemia in a 64-year male suffering with heterozygous homocysteinuria. Mohan et al. (1997) reported an isolated external iliac aneurysm associated with hyperhomocysteinemia. To date, there is only one published study in the English language that has examined the possible relationship of plasma homocysteine and AAA (Brunelli et al., 2000). Brunelli et al. (2000) investigated the prevalence of mild homocysteinemia in patients with AAA. Their data showed an association between the presence of AAA in patients selected for surgical treatment of AAA and elevated plasma homocysteine levels (see section 2.6.2.7 on thrombomodulin, pathogenesis of aneurysm).

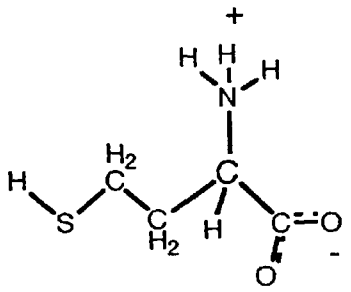


Figure 1. Structure of Homocysteine

2.3 Chemistry and Metabolism

Homocysteine $\{HSCH_2CH_2CH(N^+H_3)CO_2^-\}$ is a sulphur containing amino acid (figure-1) and is the sum of homocyst(e)ine, homocystine, and the homocysteine-cystine mixed disulphides, free and protein bound (Fortin and Genest, 1995). Hyperhomocysteinemia is defined as an increased level of fasting homocysteine or increased level of homocysteine after a loading dose of methionine. Stryer (1988) suggested that homocysteine might be considered as the by-product of the demethylation of methionine. The latter process involves the formation of s-adenosylmethionine (SAM) which serves as a methyl donor mainly in the synthesis of creatine, norepinephrine to form epinephrine, phosphatidyl-ethanolamine to form phosphatidyl-choline, cytosine to form 5-methylcytosine as well as several trans-membrane proteins.

Remethylation of homocysteine to methionine involves methylation by a complex process (Fortin and Genest, 1995) (figure-2). Methylene is donated by serine

to tetrahydrofolate derived from folic acid, to form 5`10` methylene tetrahydrofolate by the enzyme methylene tetrahydrofolate reductase which is then reduced to 5`methyl tetrahydrofolate with the release of a methyl group, by the enzyme methyl tetrahydrofolate reductase (MTHFR). The methyl group thus released is made available for remethylation of homocysteine to methionine by the enzyme homocysteine methionine methyl transferase (HMMT) using cobalamine (vitamin B_{12}) as co-factor. Thus, the process of remethylation of homocysteine to methionine is dependent on adequate availability of folic acid and vitamins B_{12}. An alternative or second pathway of remethylation involves the availability of methyl group from betaine forming di-methyl-glycine in the process. This pathway is not dependent on folic acid or vitamin B_{12}. The third alternative in the homocysteine metabolism is the formation of cysteine, via a trans-sulphuration reaction (Fortin and Genest, 1995). Homocysteine first forms cystathionine by transfer of beta carbon onto serine, with the help of cystathionine-β-synthetase, a pyridoxal phosphate dependant enzyme (PLP) that is a derivative of vitamin B_6. Cystathionine is then cleaved to cysteine and α-ketobutyric acid, by the enzyme γ-cystathionase, which is also a PLP dependant enzyme. Essentially, homocystine does not appear to have a biologic role, and is remethylated to methionine or transulphurated to cysteine. Thus, any fall in remethylation or transulphuration of homocysteine, or increased demethylation of methionine results in a hyperhomocysteinemic state.

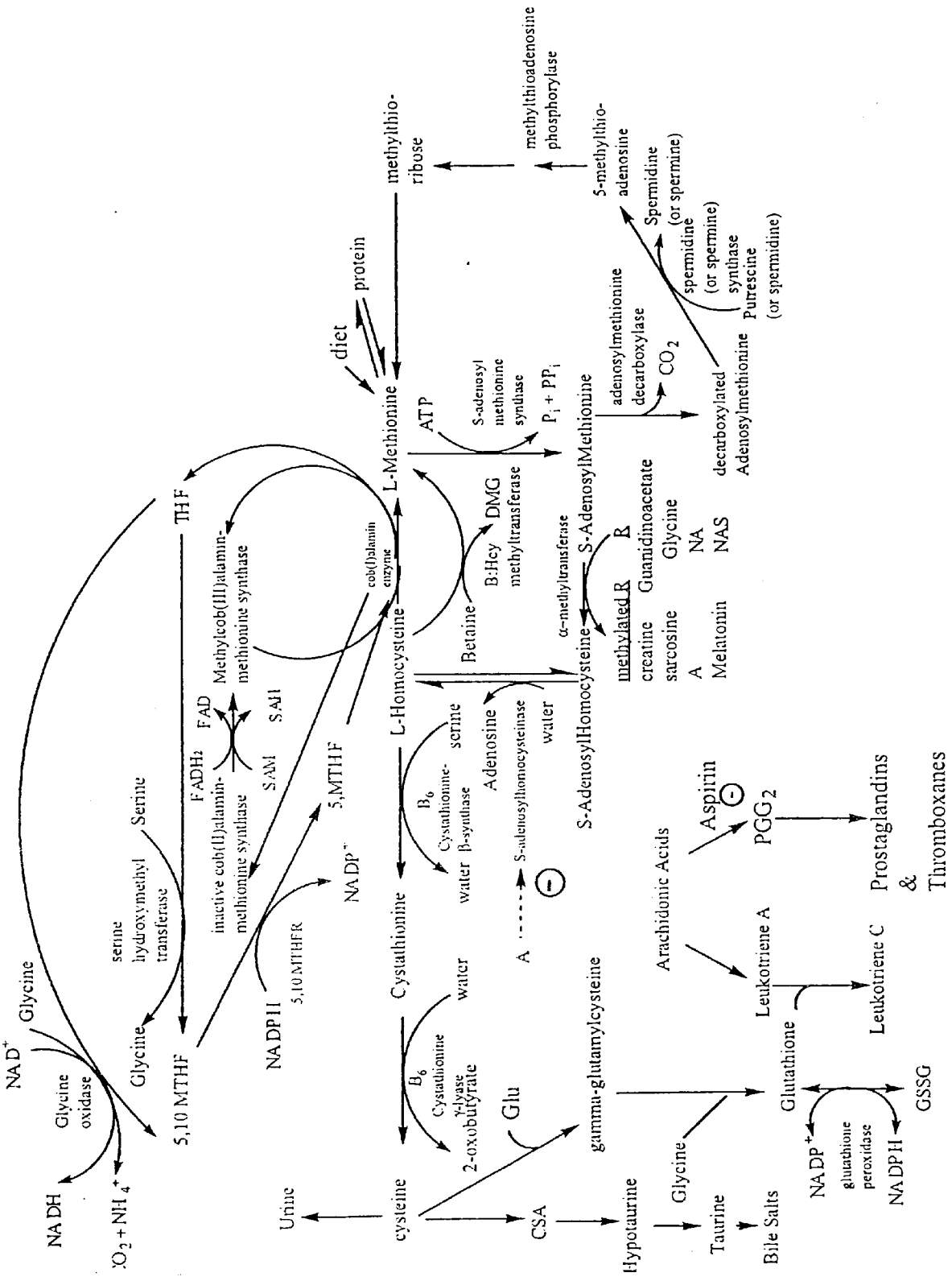


Figure 2. Biochemical pathway of homocysteine metabolism

MTHF= methyl tetra hydro folate, HMMT= homocysteine methionine methyl transferase, THF= tetrahydrofolate, SAM = s adenosyl methionine

2.4 Homocysteine and Peripheral Vascular Disease- Clinical Implications.

2.4.1 Introduction

Peripheral vascular disease is a common clinical condition. Annual mortality from peripheral vascular disease in the U.K. is estimated to be more than 2,500 and mortality from vascular disease of arteries and arterioles is estimated to be nearly 16,000 per annum (Office of Population Censuses and Surveys. 1995).

The natural history of homozygous homocysteinuria due to cystathionine synthetase deficiency demonstrates that severe hyperhomocysteinemia is a strong risk factor for vascular disease (Mudd et al., 1995; Boers, 1986). Mudd et al., 1985 also found that the occurrence of arterial occlusive diseases and thromboembolism in young adults is strikingly frequent such that 50% of untreated homozygous homocysteinuric patients under the age of 30 years may develop vascular disease. Other authors have also concluded that thrombotic and arteriosclerotic complications in homocysteinuria suggests that carriers for this condition with slightly elevated homocysteine levels might be at increased risk for vascular diseases (Wilcken and Dudman, 1989; Wilcken and Wilcken, 1976; McCully, 1969). It has been suggested that the risk factor for vascular diseases in the general population is mild hyper homocysteinemia. This is due to the more frequent distribution in the population of mild defects in methionine metabolism leading to moderately elevated blood homocysteine levels (Clarke et al., 1991; Boers et al., 1985). They also concluded that heterozygosity for classical homocysteinuria was the basis of mild hyperhomocysteinemia seen in vascular patients.

2.4.2 Homocysteine and Vascular Disease

Reports from two studies based on biochemical and pathological studies in homocysteinuric children showed that elevated blood homocysteine may cause atherosclerosis (McCully and Wilson, 1975; Mudd et al., 1995). Further studies suggested that elevated homocysteine was a risk factor for arterial and venous thromboembolism (Clarke et al., 1991; den Heijer et al., 1998). Other authors have concluded a similar outcome and reported milder increases in plasma homocysteine levels in the presence of vascular diseases specially of large and medium sized arteries, such as the coronary arteries, the iliac, femoral, and carotid arteries (Clarke et al., 1991; Genest et al., 1990; Coull et al., 1990; Brattstrom et al., 1990; Malinow et al., 1989; Brattstrom et al., 1984).

Swift and Shultz (1986) reported that a higher plasma homocysteine value was found in high risk groups (people with one or more risk factors such as hypertension, hyperlipidemia, smoking, obesity and diabetes) for coronary artery disease compared to low risk groups (people with none of the known risk factors). Genest et al. (1990) found that patients with 4 or more risk factors had higher homocysteine levels than patients with 2 risk factors. Boushey et al. (1995) indicated that elevated fasting levels of plasma homocysteine constitute a strong risk for vascular diseases. They calculated that for each increase in total homocysteine of 5 μmol/L (one SD from mean level in the normal population) there was an increment of about 40% in relative risk of coronary artery disease, a risk which is comparable with the effect of a rise of blood cholesterol of 0.5 μmol/L. This finding demonstrates comparable strengths of cholesterol and homocysteine as a risk factor for vascular diseases. Boushey et al. (1995) further suggested that an elevation in homocysteine was an independent graded

risk factor for arteriosclerotic vascular diseases. The odds ratio for coronary artery disease of a 5 μmol/L homocysteine increment was 1.6 for men and 1.8 for women. A total of 10% of the population's CAD risk appears attributable to homocysteine. The combined (men and women) odds ratio for cerebrovascular diseases was 1.5 and for peripheral arterial diseases was 1.8. Elevated homocysteine levels were as strongly related to vascular diseases, namely coronary artery disease, cerebrovascular disease, and peripheral vascular disease, as cholesterol levels and smoking; hypertension was more strongly related to risk than was elevated homocysteine. Fasting and postload (oral intake of methionine) elevations in homocysteine had independent effects on risk and when present together the net effect was multiplicative (multiple of the fasting and postload effects). The relative risk for a subject with an elevated fasting homocysteine level was only 1.6 and that for elevated postload level was only 1.5, while for a subject with both elevated fasting and postload levels, the relative risk was 2.5 (Graham et al., 1997). Other authors found that homocysteine concentrations were elevated in upto 30% patients with atherosclerosis (Clarke et al., 1991).

Wilcken & Wilcken first demonstrated that abnormally elevated plasma cysteine-homocysteine disulphide levels after methionine loading were three times more common in patients with CAD than in controls (Wilcken and Wilcken, 1976). In CAD the ratio for mean homocysteine of patients to controls varied from 1.2 to 1.3, whereas in peripheral and cerebrovascular diseases the ratio ranged from 1.5 to 1.8. The ratio of the incidence of hyperhomocysteinemia in patients vs controls ranged form 2.6 to 5.3 in CAD and from 2.2. to 9 in PVD and CVD. The authors have not shown the statistical significance of these differences though they suggest a more pronounced correlation of homocysteinemia with peripheral and cerebrovascular arterial diseases than with CAD (Kang et al., 1992). In a further study hyper homocysteinemia was found in 41.8% of patients with peripheral and cerebrovascular

arterial diseases and in 11.9 % of those with CAD (Malinow, 1991). Others have shown that moderate and intermediate hyperhomocysteinemia was present in 12- 47% of patients with coronary, cerebral and peripheral arterial occlusive diseases despite not exhibiting the systemic abnormalities characteristic of homocysteinuria. (Kang et al., 1992). Furthermore, the risk of CAD showed a dose dependent response across the entire distribution of basal and post-methionine levels of homocysteine and this effect was statistically independent of conventional factors for atherosclerosis (Refsum et al., 1998; Graham et al., 1997; Arnesen et al., 1995).

The risk of death in men and women with CAD was highly correlated with basal levels of homocysteine; mortality estimate for subjects with homocysteine levels >15μmol/L was 24.7% against 3.8% for homocysteine levels <9μmol/L over a follow-up period of 4.6 years. In addition, the more markedly elevated fasting homocysteine levels were found in patients with dialysis-dependence and end-stage renal diseases which may also contribute independently to the excess incidence of fatal and non-fatal vascular diseases (Bostom et al., 1997).

2.4.3 Homocysteine as an independent risk factor for vascular diseases

There is evidence that indicates that moderately elevated plasma homocysteine level is an independent risk factor in the development of occlusive arterial diseases (Pancharuniti et al., 1994; Stampfer et al., 1992; Clarke et al., 1991; Genest et al., 1990). A further study demonstrated a statistically significant graded increase in plasma homocysteine levels is associated with advancing age, lessening degree of physical activity, increased smoking, higher cholesterol levels and increased diastolic blood pressure (Nygard et al., 1995). Homocysteine, free and protein bound, has been

shown to be an independent risk factor in selected population such as patients with extra cranial carotid disease and ischaemic heart disease (Selhub et al., 1995; Wilcken and Wilcken, 1976). Furthermore, the level of homocysteine in vascular diseases was independent of total cholesterol, low density lipoprotein or high density lipoprotein cholesterol (Pancharuniti et al., 1994; Stampfer et al., 1992; Genest et al., 1990), diabetes mellitus (Pancharuniti et al., 1994; Stampfer et al., 1992) smoking (Pancharuniti et al., 1994; Stampfer et al., 1992), body mass index (Pancharuniti et al., 1994; Stampfer et al., 1992), age (Pancharuniti et al., 1994), and high blood pressure (Pancharuniti et al., 1994; Stampfer et al., 1992; Genest et al., 1990; Kang et al., 1986). The odds ratio for CAD was 1.6 and after adjusting for all these risk factors was 1.4. There was no significant correlation between plasma homocysteine and LDL- cholesterol and protein ($r = -0.07$, $P = 0.51$ and $r = 0.07$, $P = 0.47$,). Stampfer (1992) showed that the relative risk (RR) for myocardial infarction in US physicians for the highest 5% vs the bottom 90% of homocysteine levels was 3.1 (95% confidence interval, 1.4 to 6.9; $P= 0.005$). After adjusting for the above risk factors the RR was 3.4 (95% confidence interval, 1.3 to 8.8; $P = 0.01$). There was no correlation between plasma homocysteine and HDL-cholesterol, body mass index and diabetes ($r= -0.07$, 0.03, 0.09, $P= > 0.05$). Renal function impairment had a direct relationship to plasma homocysteine not only because excretion is impaired but largely because kidney is the site for metabolism of bulk of the plasma homocysteine (Quiroga et al., 2001). However, homocysteine remained a risk factor for vascular disease after adjustment of serum creatinine as an indicator of renal function (Wu et al., 1994). Others (Glueck et al., 1995) have also shown that homocysteine and age were positively related to atherosclerotic events. The highest risk of an atherosclerotic event was in men and was seen when homocysteine was high and high-density lipoprotein was low. After covariance adjustment for age, sex, race,

Quetelet index (body mass index), LDL, and cholesterol, Glueck et al. (1995), observed that subjects with atherosclerotic events had higher mean homocysteine and higher cystathionine than those without events. The authors suggested that homocysteine may be usefully measured in patients with or without major conventional risk factors for atherosclerosis who nevertheless have severe premature atherosclerosis that could be due to hyperhomocysteinemia.

Until further evidence emerged (Pancharuniti et al., 1994; Stampfer et al., 1992; Genest et al., 1990; Kang et al., 1986) that homocysteine is an independent risk factor in the genesis of atherosclerosis, some authors (Mudd et al., 1981) had argued that hyperhomocysteinemia had given rise to a plethora of in vitro experiments and pathophysiogical hypothesis to explain the dramatic increase in atherosclerotic vascular diseases and in thromboembolism observed in patients with the severe inborn form of the diseases. They claimed that the epidemiological finding of increased homocysteine in patients with premature atherosclerosis had not yet received an indisputable explanation for the mechanisms involved. Moreover, they also indicated that homocysteinuric obligate heterozygotes, although presenting with high levels of plasma homocysteine levels do not have increased incidence of vascular diseases (Mudd et al., 1981). However, there were two main limitations of this study. First, because the cardiovascular diagnoses were arrived at by the questionnaire method, the validity of these diagnoses is subject to more uncertainty than would have been the case had direct physical and electrocardiographic examination been possible. Second, with a 20% loss in response rate, some unidentified participation bias may have possibly been operating. Third, presence of a more subtle interaction between homocysteine and diet such as vitamins and protein could not be ascertained because the subjects dietary habits were not known. Furthermore, their own data suggested an

association between elevated homocysteine and haemostatic disorders, and higher plasma homocysteine in patients vs controls. Several studies published subsequently (discussed in this section earlier) and the meta-analysis by Boushey et al. (1997) it is now known that homocysteine is an independent risk factor in the genesis of vascular diseases.

2.5 Pathogenesis

2.5.1 Atherosclerosis, endothelial damage, smooth muscle hyperplasia and platelet activation

Homocysteine may modulate some aspects of atherogenesis, vascular diseases and thrombosis (figure-3). A number of studies reviewed by Boushey et al. (1995) have linked elevated plasma homocysteine levels to atherosclerotic vascular diseases affecting coronary, carotid and peripheral arteries. Several mechanisms of homocysteine induced atherosclerosis have been proposed (McCully, 1969; Ueland et al., 1992). These include endothelial dysfunction, smooth muscle cell hyperplasia and increased formation of oxidised lipids. Ross et al. (1986) postulated that endothelial cell (EC) injury initiated atherosclerosis. High homocysteine levels may cause endothelial damage (Matthias et al., 1996; Harker et al., 1983), affect platelet function and coagulation factors (Lentz et al., 1996; Harker et al., 1976) and promote LDL oxidation (Heinecke et al., 1993). Substances such as lipids and homocysteine initiates endothelial injury which promotes platelet adherence to endothelium and release of platelet constituents, mainly the epidermal growth factor and the platelet derived growth factor (PDGF). These factors induce smooth muscle cells to migrate into the tunica intima of the arteries where they replicate to form connective-tissue

matrix. The connective tissue along with macrophages, cholesterol and lipids and calcium deposition form fibrous plaques that causes atherosclerosis in the vessel wall.

Chambers et al. (1999) observed that elevated levels of homocysteine concentration are associated with rapid onset endothelial dysfunction, which is mediated through oxidant stress mechanism and can be inhibited by antioxidants such as vitamin C. Animal experiments and in-vitro studies of cultured endothelial cells have given support to the hypothesis that homocysteine in excess induces endothelial cell injury and thereby initiates premature atherosclerosis (Harker et al., 1976; De Groot et al., 1983; Wall et al., 1980). These studies revealed that cultured endothelial cells that are derived from obligate heterozygotes are deficient in cystathionine beta synthetase required for homocysteine metabolism and are more susceptible to homocysteine mediated injury than normal cells. Harker et al. (1976) chemically induced injury in the arteries in baboons as early as 6 days after the beginning of a constant intravenous infusion of homocysteine that resulted in focal loss of arterial endothelial cells (Harker et al., 1976). The thoracic and abdominal aorta, iliac and femoral artery was stained in silver nitrate and viewed under light and electron microscope. This revealed that approximately 10% surface area of the endothelium was missing. There was also a 50% decrease in platelet survival, which mimicked those observed in familial homocysteinuria in man. Several authors have demonstrated that atherosclerosis was also common in patients heterozygous for homocysteinemia, and in those with high homocysteine levels due to reduced renal function, folate and vitamin B_{12} deficiency (Dudman et al., 1993; Stampfer et al., 1992; Clarke et al., 1991; Genest et al., 1990; Mudd et al., 1995).

2.5.2 Nitric oxide, Glutathione peroxidase, Lipid peroxidase

Although the mechanism by which hyperhomocysteinemia evokes endothelial dysfunction are not well understood it is proposed that this action is mediated by impaired production of nitric oxide (Stamler et al., 1993). In vitro studies (Nishinaga et al., 1993) showed that initial exposure of cultured endothelial cells to homocysteine leads to formation and release of nitric oxide, S-nitrosothiols, and S-nitrosohomocysteine, substances with potent vasodilator and platelet inhibition properties. However, with continued exposure the oxidative effects of homocysteine predominate leading to reduced production or inactivation of nitric oxide (Nishinaga et al., 1993; Stamler et al., 1993). Stamler and Slivka (1996) suggested mechanism for homocysteine-induced vascular lesions. They include hypersulphation of connective tissue proteoglycans, effects on platelet aggregation and survival, an increase in LDL and in LDL oxidation, which leads to formation of lipid peroxides, and direct endothelial damage by reduced oxygen supply. Homocysteine also inhibits glutathione peroxide which contributes to endothelial cellular defence mechanism against oxidative stress, by catalysing the reduction of both H_2O_2 and lipid peroxide to less toxic alcohols. Stamler et al. (1993) also found that impaired availability of nitric oxide made the endothelial cells vulnerable to unopposed homocysteine-mediated oxidative damage.

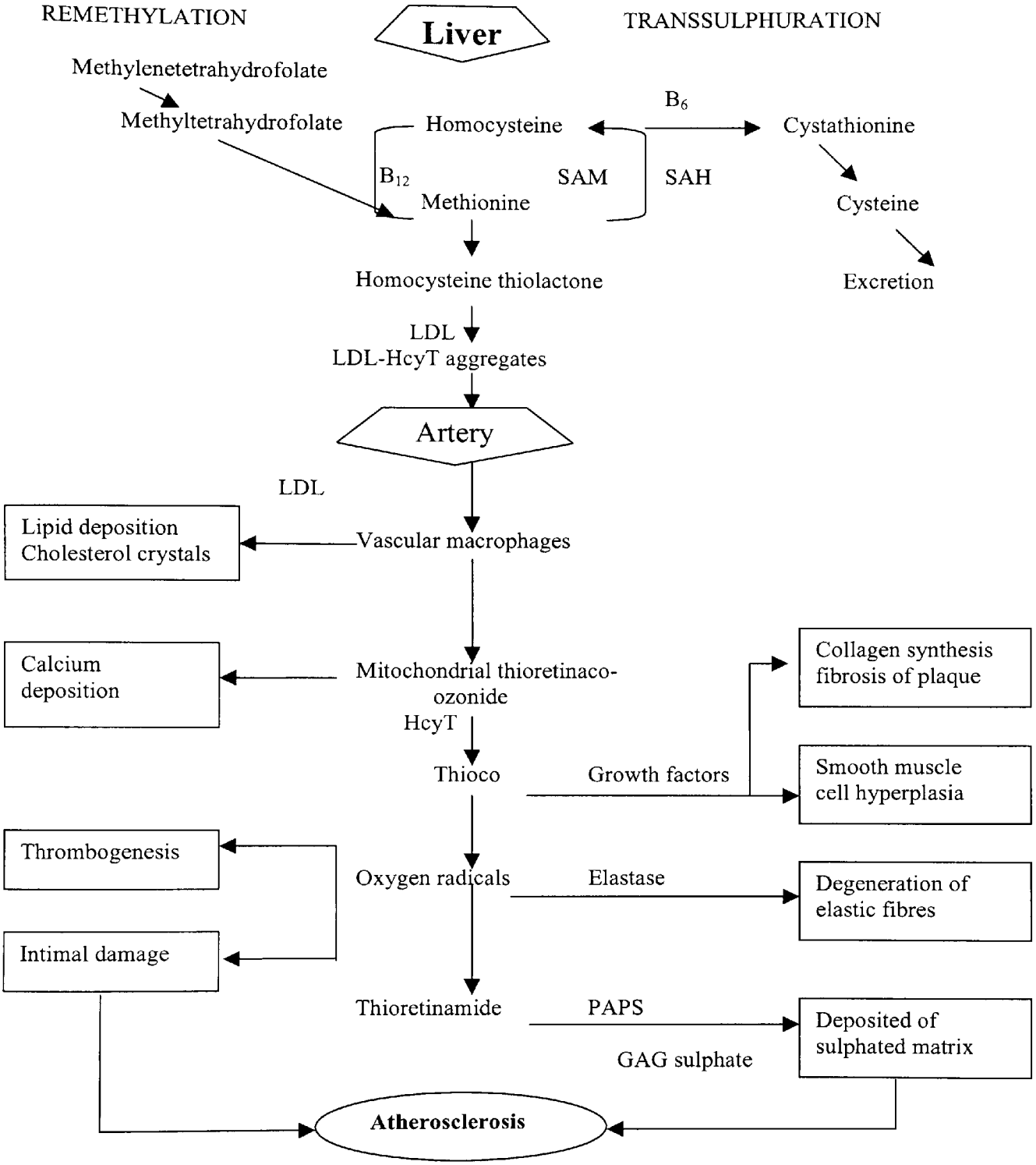


Figure 3. The role of homocysteine in the pathogenesis of atherosclerosis.

Abbreviations: B_{12}, cobalamin; B_6, Pyridoxal phosphate; SAM, S-adenosyl methionine; LDL, Low density lipoprotein; HcyT, homocysteine thiolactone; PAPS, Phosphoadenosine phosphosulphate; GAG, glycosaminoglycans.

2.5.3 Hydrogen peroxide (H_2O_2)

Sulphur-containing amino acids such as homocysteine disrupt endothelial cell in vitro by a mechanism that involves generation of hydrogen peroxide in a copper catalysed reaction that causes auto-oxidation of homocysteine (Wall et al., 1980; Starkebaum and Harlan, 1986; Loscalzo, 1996). The H_2O_2 induces DNA degradation, and inhibits DNA synthesis in endothelial cells in a dose dependant manner (Loscalzo, 1996; Blundell et al., 1996) Panganamala et al. (1986) and Saez et al. (1982) further confirmed that homocysteine-induced endothelial cell detachment was prevented by catalase but not by superoxide dismutase, which would suggest that hydrogen peroxide plays a role in mediating injury.

2.5.4 Coagulation Factors- Prostaglandin I_2 (PGI_2), Thromboxane

Homocysteine induces chemical injury to cultured endothelial cells due to modification of arachidonic acid metabolism. It seems that homocysteine participates in the generation of hydrogen peroxide, which interacts with the synthesis of arterial prostacyclines such as prostaglandin I_2 (PGI_2) (Starkebaum and Harlan, 1986; Saez et al., 1982; Graeber et al., 1982). The effect of homocysteine on PGI_2 synthesis seems to be dependent on the oxidation of homocysteine and the subsequent generation of hydrogen peroxide. Once the production of prostacyclines such as PGI_2 is suppressed the normal haemostatic balance between it and the increased platelet thromboxane A_2 is lost, which promotes coagulation.

2.5.5 Tissue Factor

Tissue factor (TF) which has procoagulant activity has been shown to increase with elevated levels of homocysteine. Homocysteine increased HUVE (Human umbilical vein endothelial) cell tissue function activity in a concentration and time-dependent manner, with a seven-fold increase in tissue function activity seen with 10mmol/L homocysteine. A higher concentration of homocysteine induced HUVE cell cytotoxity with cell retraction and detachment. Moreover, a low concentration of homocysteine similar to those found in patients with homocysteinuria, increased TF activity by 25 to 100% (Fryer et al., 1993).

2.5.6 Antithrombin III, Factors V, VII, VIII, Factor 8C, Protein-C, Thrombomodulin, Tissue Plasminogen

In patients with severe hyperhomocysteinemia reduced levels of factors VII and antithrombin III were found (Palareti and Coccheri, 1989). These were correctable by administration of folate and pyridoxine (Rodgers and Kane, 1986). Homocysteine was also found to reduce protein C activation thus neutralising its protective anticoagulant activity (Rodgers and Conn, 1990). Hayashi et al. (1992) suggested that homocysteine can be thrombogenic by displacing endothelial cell protein C from thrombomodulin. Boushey and Shirley (1995) further supported this view in a quantitative assessment of plasma homocysteine as a risk factor for vascular disease. They assessed the effects of homocysteine on vascular haemostatic properties and concluded that decreased

thrombomodulin cell surface expression and inhibition of protein C activation probably contributed to the development of thrombosis.

Rodgers et al. (1986) have demonstrated that in terms of thrombotic mechanism homocystine activates factor XII and endothelial cell factor V. Harpel et al. (1992) observed that homocysteine enhances the binding of an atherogenic lipoprotein to fibrin. This occurs at the expense of binding of tissue plasminogen activator to fibrin, thus shifting the balance to a coagulant state. Stamler and Slivka (1996) suggested the mechanism for hyperhomocysteinemia induced vascular lesions. They are hypersulphation of connective tissue proteoglycans, effects on platelet aggregation and survival, an increase in LDL and in LDL oxidation, and direct endothelial damage by reduced oxygen species (see section on nitric oxide). In the presence of fasting hyperhomocysteinemia, factors VIIIc and Von Willebrand were significantly increased and thrombin-antithrombin complexes were more elevated (Freyburger et al., 1997). Moreover, when post-methionine load homocysteine was increased, alterations in fibrinolytic parameters were more pronounced. These alterations were associated with significantly higher levels of coagulation activation markers. These results demonstrate that in mild hyperhomocysteinemic patients with vascular diseases, a trend towards higher haemostatic alterations occur than in normohomocysteinemic patients. Thus the pathogenesis involves endothelial damage, platelet activation, adhesion and aggregation, smooth muscle proliferation and endothelial-leukocyte interaction (Murad et al., 1979; Upchurch et al., 1996; Luscher et al., 1996).

The consequences of athero-thrombosis depend on the size and the location of the arteries affected. Infarctions may result. Venous thrombosis is also common and may occur in vena cava, cerebral sinuses, and/or peripheral veins. Emboli from these

thrombi may be found in various organs. Some thrombi may be organised and recanalised. Intimal hyperplasia and fibrosis may cause the arterial lumen to be narrowed. The arterial walls may be dilated, stretched thin, or thickened forming aneurysms. Certain nutritional hyperhomocysteinemias may also be associated with the development of arteriosclerotic lesions, irrespective of which nutrient is involved, hyperhomocysteinemia is likely to be the common denominator

2.6 Aneurysms

2.6.1 Introduction

The prevalence of abdominal aortic aneurysm is about 4% in the elderly U.K. population of >65 years (Office of Population Censuses and Surveys. 1985). However, 5000 people in the UK die every year because of AAA and associated complications (Office for National Statistics. 1995). Whereas mortality is less than 6% in patients undergoing elective surgery for AAA, it rises to more than 80% when operated on an emergency basis (Campbell et al., 2001). Hence, it is important that the risk factors associated with AAA be detected as early as possible. Although the association of hypertension (29%), smoking (28%) and previous history of MI and angina (16%) with AAA have been well documented (Campbell et al., 2001), the biochemical markers of AAA are yet to be evaluated fully. However, certain biochemical markers have been implicated in the pathogenesis of vascular diseases and aneurysms as detailed below:-

2.6.2 Pathogenesis

2.6.2.1 Hydrogen peroxide

A mechanism has been suggested whereby elevated levels of homocysteine could injure endothelial cells through copper catalysed generation of hydrogen peroxide (Starkebaum and Harlan, 1986). Homocysteine-induced endothelial cell

damage has been recognised by some authors (Panganamala et al., 1986; Saez et al., 1982) . Please see details on hydrogen peroxide in section 2.5.3.

2.6.2.2 Cytokines

Rhode et al. (1999) examined plasma concentration of Interleukin-6 (IL-6), (inflammatory cytokine that seems to play a pivotal role in the acute-phase response) abdominal aortic diameter and plasma homocysteine among 120 subjects referred for transthoracic echocardiogram. In multivariate regression analysis adjusted for age, hypertension, diabetes, smoking, ischaemic heart disease and hyperlipidaemia the significant correlates of aortic diameter were plasma IL-6 ($r = 0.285$; $P = 0.03$), total homocysteine ($r = 0.241$; $P = 0.01$) and Serum Amyloid A (SAA) ($r = 0.274$; $P = 0.004$). However, unlike other markers of inflammation such as IL-6 and SAA, there was no association with C Reactive Protein (CRP) ($r = 0.03$; $P = 0.75$). The reason for the lack of association between CRP levels and aortic size are unclear, since clinical information provided by CRP and SAA are expected to be similar. In this cohort, CRP may be insufficiently specific to uncover association between initial inflammatory processes and vascular remodelling. Differences in the clearance of CRP and SAA could partially explain these findings. In addition, the associations may not have been detected because of the small sample size. However, their data support a role for chronic inflammation in the progression of asymptomatic aortic disease. Their studies suggest that cytokine-induced tissue inflammation may participate in the pathogenesis of AAA.

2.6.2.3 Genetic basis

Frost et al. (1995) proposed the C677T polymorphism in the MTHFR gene as a risk factor for vascular disease. The genotype (the genetic information defining the phenotype is called the genotype; an observed trait is referred to as phenotype) distribution of C677T MTHFR consists of a combination of TT, TC, CC. Tsukahara et al. (2000) showed that in the female population the frequency of TT genotype in patients with coronary aneurysm was significantly lower than in patients without this manifestation. TT genotype may protect female Kawasaki disease patients against aneurysm formation and predispose male Kawasaki disease patients to severe coronary complications. Miner et al. (1997) opined that individuals homozygous for thermolabile variants of MTHFR have elevated plasma homocystine levels. Since it arises from a common mutation 677 C to T, this may represent a genetic factor for the vascular disease. The mutation occurs in 5,10-MTHFR gene i.e., a C to T substitution at nucleotide 677 or valine to alanine substitution which leads to reduced MTHFR activity and moderately high plasma homocysteine levels (Miner et al., 1997; Frosst et al., 1995).

Homozygous homocysteinuria, the most common genetic disorder of transulphuration is associated with elevated plasma concentration of homocysteine and homocystine. Multiple clinical abnormalities, life-threatening thromboembolism and several instances of vascular aneurysm have also been documented (Colwell et al., 1991) (see section 2.2).

2.6.2.4 Protein C

Rodgers et al. (1990) demonstrated that homocysteine causes endothelial dysfunction at several levels by depressing activation of protein C which has an antithrombotic activity. Their data suggested that homocysteine-treated vascular endothelium induces activation of factor V which in turn reduces endothelial cell protein C activation. This leads to coagulation abnormalities and a tendency towards thrombosis.

2.6.2.5 Nitric oxide and Lipid peroxides

Stamler et al. (1993) found that homocysteine in vitro, impairs production of nitric oxide and promotes lipid peroxidation. McCully et al. (1971, 1994) have shown that homocysteine-induced disturbance in oxidative metabolism leads to intimal injury, activation of elastase & increased deposition of calcium.

2.6.2.6 Serine elastase

Jourdheuil-Rahmani et al. (1997) noted that homocysteine stimulates the synthesis of serine elastase in arterial smooth muscle cells. Campa et al. (1987) found a markedly decreased elastin content (8% dry weight of aneurysmal aorta vs 35% dry weight of normal aorta) and a high elastinolytic activity in section of the aorta of patients of abdominal aortic aneurysm. Bescond et al. (1998) have shown that in animal models homocysteine induces elastolysis in arterial media by the activation of matrix metalloproteinases-2 (MMP-2).

2.6.2.7 Thrombomodulin and MTHFR-TT genotype mutation

Brunelli et al. (2000) tried to evaluate homocysteine and thrombomodulin plasma levels in patients with AAA. They also evaluated the prevalence of C677T MTHFR mutation in patients with AAA and the influence of this mutation on plasma homocystine levels. They demonstrated that patients with AAA had significantly higher levels of plasma homocysteine in comparison to control. Moreover, the subgroup of patients with AAA who did not show evidence of atherosclerosis also showed plasma homocysteine levels to be significantly higher, suggesting that elevated levels of homocysteine can induce aneurysm formation without actually causing atherosclerosis. Furthermore, in patients with AAA, a larger aneurysm size was detected in hyperhomocysteinemics than in those with normal plasma homocysteine level. They also showed that plasma thrombomodulin levels were significantly higher in hyperhomocysteinemics than in normal homocysteinemics. In patients with aneurysm a significant correlation was found between homocysteine and thrombomodulin. They suggested that homocysteine may interact with the aortic wall and induce both elastolysis and cause endothelial damage in these patients. They further demonstrated a significant correlation between plasma homocysteine and MTHFR TT genotype in patients with AAA.

2.6.2.8 Inhibition of anticoagulation mechanism

Some authors have suggested that the presence of a reduced form of homocysteine may induce endothelial injury by inhibiting endothelial anticoagulant mechanism (Nishinaga et al., 1993; Starkebaum and Harlan, 1986). Colwell et al.

(1991) suggested an association between hyperhomocysteinemia and multiple aneurysms but said that the exact mechanism in aneurysm formation is not clear.

It is therefore concluded that homocysteine is an independent graded risk factor for atherosclerosis in the general population. Atherosclerosis is an arterial disease that is recognised to be the chief cause of death in the USA and Europe, the disease progresses insidiously for many years before symptoms develop, making it difficult to follow the early development of the diseases in individual patients. Identification of risk factors by early investigation, and their modification has the potential to modify vascular disease progression. Finally, the association between abdominal aortic aneurysm and plasma homocysteine is largely unexplored.

2.7 Peripheral Vascular Disease (PVD)

Peripheral vascular disease, as the name implies is the disease of the arteries of the peripheries. In a vast majority of patients the disease involves the lower limbs but can also affect the upper extremity. It is commoner in those with risk factors such as increasing age (Leng et al., 1996), smoking (Hiatt et al., 1995), hypercholesterolemia and hyperlipidemia (Murabito et al., 1997; Ueland and Refsum,1989) hypertension and diabetes (UK Prospective Diabetes Study Group, 1998). Since PVD is more common in diabetics, it is worth noting that 30% of diabetics will eventually require surgical intervention and 10% will require amputation for PVD, besides the fact that in diabetic patients amputation rate is seven times higher than in non-diabetics (Rosenfield and Isner 1998; McAllister 1976). There is evidence to suggest that hyperhomocysteinemia may also be risk factor for peripheral vascular disease (Boushey et al., 1995; Ueland and Refsum 1989).

The annual mortality from the vascular disease of the arteries and arterioles is estimated to be nearly 16,000 per annum (Office of Population Censuses and Surveys. 1995) in the U.K., of which 2,500 is from PVD. The rest is attributed to mortality from aortic aneurysms, other aneurysms, unspecified atherosclerosis, thrombosis, embolism and various arteritis. This does not include figures form ischaemic heart disease and cerebrovascular which comprise of over 110, 000 mortality per year.

Since the incidence of the peripheral vascular disease in the lower limbs far exceeds that in the upper limb (Haimovici, 1950), it would seem appropriate to imply henceforth, the acute limb ischemia (ALI) and chronic limb ischemia (CLI) as the disease state in the lower limbs unless stated otherwise.

2.7.1 Classification

Peripheral vascular disease can be broadly classified into two categories based on the clinical presentation:

1.Acute limb ischemia (ALI) and

2.Chronic limb ischemia (CLI)

2.7.2 **Acute limb ischemia** (ALI)

The incidence of this disease tends to occur in about 20 in 25,0000 population in the U.K. each year (Office of Population Censuses and Surveys, 1995). It is therefore, a relatively uncommon condition. The incidence is however greater in the elderly and appears to be increasing.

The causes of the acute limb ischemia can be summarised as follows:

2.7.2.1 Thrombosis

Thrombosis can be due to:

Atheromatous stenosis

Popliteal aneurysm

Graft stenosis

Cardiac failure

Thrombotic states

(modified from Beard and Gaines, 1999).

The commonest cause of ALI is thrombosis at an atherosclerotic arterial stenosis, typically of the superficial femoral and popliteal artery. This is found to be commoner in the older patients as the incidence of atherosclerosis increases with age (Leng et al., 1996).

2.7.2.2 Embolism

Embolism can originate from:

Ischemic heart disease

Cardiac arrhythmia

Mural thrombosis

Valvular vegetations

Atrial myxoma

Aortic aneurysm

Atherosclerosis plaques

(modified from Beard and Gaines, 1999)

Atrial fibrillation as a result of ischemic heart disease is one of the commonest cause of emboli in the western world although in the developing countries rheumatic heart disease may still be the commonest cause despite a decline in its incidence. Emboli usually lodge at the vessel bifurcation, the commonest site in the lower limb being the bifurcation of the common femoral artery. This is due to the decreasing diameter of the vessel as the embolus passes down the lumen distally, obstructing at the point where the vessel lumen is too narrow for the embolus to squeeze through.

2.7.2.3 Others

Other causes of ALI include:

Aortic dissection

Arterial trauma

External compression

Compartment syndrome

Popliteal entrapment

Cystic adventitial disease

(modified from Beard and Gaines, 1999)

2.7.2.4 Symptoms of acute limb ischemia

The classical symptoms and signs of ALI, '6P's' are:

Pain

Pallor

Pulselessness

Parasthesia

Paralysis

Perishing with cold

(modified from Murie JA 2000)

These symptoms are suggestive of *severe* ALI with the leg pale and cold with mottled appearance, which may initially blanch on pressure. Presence of sensory-motor loss is an indication for urgent surgery. Non-blanching and paralysis are signs of irreversible vascular injury resulting in limb loss that require amputation.

Mild to moderate ALI may be result of thrombotic stenosis or an incomplete arterial occlusion. There is invariably an element of collateral circulation distal to the level of occlusion. *Collateral circulation* is the leash of smaller vessels around the main obstructed vessel, which may reconstitute and unite with the main vessel distal to the occlusion.

It is important to distinguish between emboli and a thrombosis as the approach to treatment and prognosis differs. *Emboli* can be caused by cardiac arrhythmia usually secondary to ischemic heart disease, with acute onset of severe symptoms, an affected pale or white leg with normal pulses in the contralateral leg and demonstration of normal arteries on arteriogram. *Thrombosis* can be caused by atheromatous stenosis or aneurysm, usually preceded by history of claudication, less acute onset of symptoms, a dusky affected foot on examination with absent or weak contralateral pulses, and confirmation of diseased arteries on arteriogram.

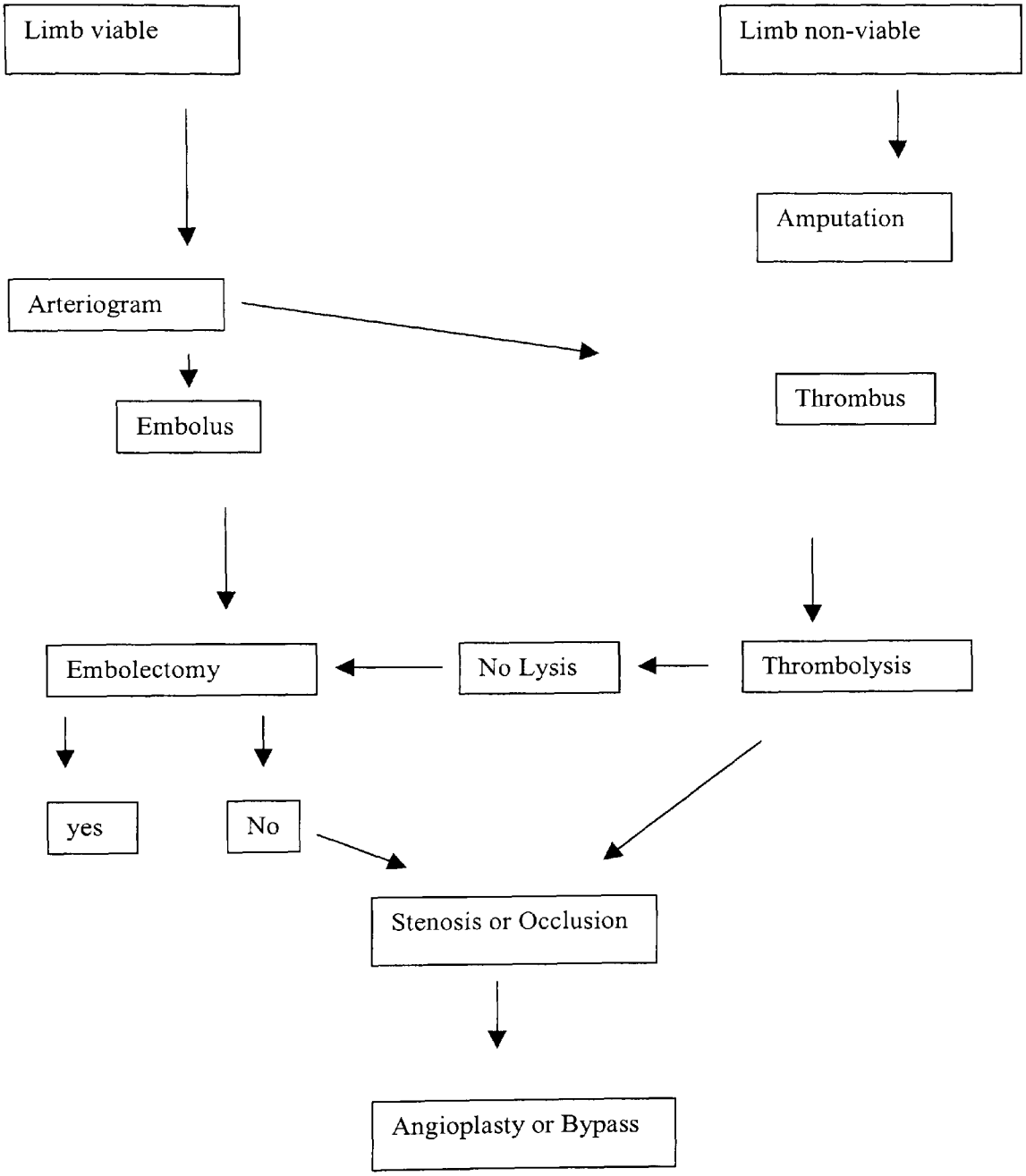
2.7.2.5 Management

Once a diagnosis is made on clinical grounds patients should receive unfractionated heparin as a bolus intravenous dose of 5,000-10,000 units followed by 1000 units/hour of intravenous infusion. Adequate analgesics and intravenous fluids should be started to prevent propagation of the thrombus. Necessary blood tests such as full blood count (FBC), urea electrolyte and creatinine (UEC), blood glucose, erythrocyte sedimentation rate (ESR), group and save, and clotting screen should be requested along with chest x-ray and electrocardiogram (ECG), to assess and optimise

the preoperative status of the patient and to have a baseline for postoperative monitoring.

The diagnosis should ideally be confirmed on arteriography. If an embolus is confirmed, embolectomy should be performed. For a thrombus, thrombolysis (dissolving of the clot) can be carried out. The treatment plan is set out in the flow chart below in figure-4.

Figure-4. Flow chart for the management of acute limb ischemia. (modified from Beard and Gaines, 1999).



2.7.3 Chronic Limb Ischemia (CLI)

Chronic limb ischemia is a result of slowly progressive arterial occlusion caused by degenerative arterial disease. In a vast majority of the patients with PVD, atherosclerosis is the chief cause of the occlusion. 4 - 8% of the population between the age of 55 to 74 have chronic limb ischemia with the incidence rising to 16% when higher age groups are included (Leng et al., 1996).

2.7.3.1 Symptoms and Signs of CLI

CLI is characterised by intermittent claudication of the calf and/or the thigh muscles with absent or weak foot pulses except in conditions such as diabetes where the pulses may be palpable. Claudication of the calf, thigh or buttock implies disease in the femoropopliteal, ileofemoral and aortoiliac segment of the artery respectively. Physical signs may include a loss of hair, shiny, thin and pale skin, muscle wasting, unhealthy nails and bruit on auscultation distal to the stenosis or diseased segment in the artery.

2.7.3.2 Management

The diagnosis is usually confirmed by measuring the ankle brachial ratio or ankle brachial pressure index *(ABPI)* (see section on ABPI in methodology). This is the ratio of the blood pressure measured in the lower limbs to that in the upper limb. An ABPI of <0.9 in the presence of symptoms and signs is sufficient to confirm the diagnosis (Carter, 1968; Yao, 1969; Yao, 1970). Where pulses may seem normal at rest, the chronic ischemia in a limb can be unmasked by means of an *Exercise Stress*

Test, where the ABPI in such a limb falls after exercise. The physiological principle that explains this phenomenon can be explained as follows. In the presence of occlusive lower limb arterial lesions, blood is diverted through high resistance collateral vessels or pathways. Although the collateral vessels provide adequate flow to the resting extremity with only a modest reduction in ankle pressure i.e. modest fall in ABPI, the capacity of these collaterals to increase blood flow during exercise is limited. Pressure gradients that are minimal at rest are accentuated by the stress test, thus unmasking less severe degree of arterial disease.

Other tests such as *Duplex ultrasound scan* or *Arteriography* can be carried out when the suitability of intervention has to be decided.

The medical treatment includes modification of risk factors by changing the life style. Graded exercise programmes may also bring about improvement in symptoms and signs. Failing this, intervention in the form of vascular or endovascular repair is carried out.

Endovascular repair consists of angioplasty or stenting and vascular repair consists of endarterectomy or bypass using autologous vein or prosthetic grafts.

Critical limb ischemia is an extreme type of CLI, which comprises multiple level of arterial disease, an ABPI of ≤ 0.5 and clinically presenting with rest pain in the leg with or without ulceration and gangrene. Revascularisation of the limb by vascular or endovascular surgical intervention may be needed for limb salvage.

2.7.4 Abdominal Aortic Aneurysm

2.7.4.1 Introduction

Aneurysm is a localised dilatation of an artery. When an aneurysm occurs in the central artery of the body called the aorta, it is known as an aortic aneurysm. An aortic aneurysm located in the abdominal part of the aorta is thus referred to as an abdominal aortic aneurysm. The normal diameter of the abdominal aorta in adults is 2 cm in either the anterio-posterior or transverse axis. By definition the abdominal aorta is said to be aneurysmal if the diameter is 3cm or greater. Others have suggested that an increase of 50% in the size of aorta should be classed as an aneurysm since the normal diameter of abdominal aorta can vary with age, sex and body mass index (Johnston et al., 1991; Lindholt et al., 1997). Abdominal aortic aneurysms usually arise below the renal arteries when it is called the infra-renal abdominal aortic aneurysms and are amenable to repair. The supra-renal abdominal aortic aneurysms are rare and more difficult to repair.

Abdominal aortic aneurysm is prevalent in 5% of adult males above the age of 65 years (Collin et al., 1988). The incidence of AAA has been steadily increasing over the last 30 years due to increased detection and awareness of the disease and also due to the increasing number of elderly population (Fowkes et al., 1989). Death from AAA ranks thirteenth amongst the common causes of death in the United Kingdom (Office of Population Censuses and Surveys. 1995). The total number of deaths from all aneurysms (thoracic, abdominal and other aneurysms) in the United Kingdom was 9252 in the 1993 reported in the Office of Population Censuses and Surveys, 1995. Of these, 3883 deaths (2848 men and 1035 women) were from the ruptured abdominal aortic aneurysms and 572 from the non-ruptured abdominal aortic aneurysm i.e.

elective surgical repair. Ultrasound scan of the abdomen is used to screen and detect asymptomatic or non-ruptured AAA, and the recommendation for surveillance is bi-yearly for aneurysm < 4 cms and yearly for those ≥ 4 cms (Cook and Galland 1996).

2.7.4.2 Aetiology

The well known causes for an aneurysm are:

Atherosclerosis

Inflammation

Infection

Trauma

Connective tissue disorders

(modified from Wyatt MJ, 1999)

2.7.4.3 Clinical features

Most abdominal aortic aneurysms are asymptomatic and are discovered as an incidental finding on routine ultrasound scan (USS) of the abdomen for other reasons, during screening for abdominal aortic aneurysm on USS or less commonly during routine clinical abdominal examination in patients or on computerised tomography scan (CTScan) (Davies et al., 1999).

AAA can cause symptoms from the contained thrombus or from rupture of the aneurysm itself. A thromboembolic phenomena related to the AAA presents with ALI of the lower limb.

A rupture must be suspected in a patient with abdominal and or back pain with features of hypovolemic shock namely hypotension, tachycardia and pallor. Ruptures can be intraperitoneal in which case these signs are more dramatic. Though all these signs are present in a retroperitoneal rupture the outcome is better because the rupture is contained inside the less yeilding retroperitoneal space.

Most AAA, except for the small ones, can be bimanually palpated as a pulsatile and expansile mass in the abdomen above and slightly to the left of the umbilicus. The diagnosis is usually confirmed by ultrasound scan of the abdomen. It is widely popular as the first investigation of choice because it is safe, widely available, simple to use, non-invasive and able to diagnose the presence of an AAA in the vast majority of patients with reported sensitivity around 97% in some centres (Smith et al., 1993). In doubtful cases however, (CT scan - with or without contrast), a Spiral CT scan or a magnetic resonance imaging scan (MRI scan) usually confirms the presence or absence of an AAA.

2.7.4.4 Management

There is now a general consensus amongst vascular surgeons that abdominal aortic aneurysm of $\geq$ 5.5 cm should undergo repair following the results of the UK Small Aneurysm Trial (1998). Other indications for an AAA repair include:

-An expansion of 1cm or more/year

-Development of symptoms such as abdominal pain/back pain or abdominal tenderness.

Repair can be surgical or endovascular.

Surgical repair

Surgical repair of an AAA is a major operation. This is widely carried out by the conventional laparotomy. Some surgeons however, opt for a retroperitoneal approach. The principle of the operation involves a midline laparotomy, proximal clamp control on the aorta, distal clamps on the common iliac arteries, opening of the aneurysmal sac, and inlay of a prosthetic graft, commonly a dacron graft using non-absorbable sutures, commonly prolene of size 2/0 or 3/0. The mortality from an elective repair is reported to be around 6%, but for ruptured abdominal aortic aneurysm repair the mortality has been reported to be around 80% (Galland, 1998; Fowkes et al., 1989; Johansson and Swedenborg, 1986; Basnyat et al., 1999).

Endovascular repair

This is still in the trial stage and is carried out in selected centres. The principle of this technique involves assessment of the suitability of the aortic aneurysm for endovascular repair, access into the aorta via common femoral artery, insertion of a predesigned prosthesis (commonly a bifurcated graft) and its lodgement into the aortic sac. This is done under radiological control in an elective setting, and is a joint endeavour between surgeons and radiologists. Early results suggest that 30-day mortality is less than the conventional repair but problems with 'endoleak' and graft migration need to be resolved. (Professor Greenhalgh, London, - personal communication). These figures for mortality and morbidity will be available once the trail is complete.

Following the results of the UK Small Aneurysm Trial (1998), there is also now a greater consensus amongst vascular surgeons that abdominal aortic aneurysm less than 5.5 cms should be followed-up by serial ultrasound scans, unless there are other indications for operation. The frequencies with which they are followed-up are as follows:

< 4 cm	yearly follow up with USS
4 - < 5cm	6 monthly follow up with USS
5 - < 5.5 cm	3 monthly follow up with USS
5.5 cm or >	operate

In summary, PVD and AAA are common in modern society, in the middle-aged and the elderly population. Their aetiology is complex and multi-factorial. Prevention of disease progression could be achieved by reduction of risk factors. Open surgical repair, radiological intervention or a combined approach are the modalities used in the treatment of these conditions.

2.8 Vitamins and vascular diseases

2.8.1 Introduction

Serum levels of several vitamins have been shown to affect the level of total plasma homocysteine (Woodside et al., 1998; Selhub et al., 1993). Folate and vitamin B_{12}, are required in the conversion of homocysteine to methionine while vitamin B_6 is required in the conversion of homocysteine to cysteine. Vitamin A helps in the conversion of homocysteine thiolactone to thioretinamide which in turn is converted to sulphate (IV) in the presence of Vitamin C (ascorbic acid). Mild elevation in plasma homocysteine can be caused by deficiencies in serum folate, vitamin B_{12} or vitamin B_6.

2.8.2 Biochemistry

2.8.2.1 Folic Acid

One-carbon tetrahydropteroylglutamate (folates), are involved in many biochemical pathways that are necessary for growth, development and homeostasis of humans. Unfortunately, humans are unable to synthesise folate *de novo*. A deficiency of this vitamin is associated with neural tube defects and cardiovascular disease (Krumdieck et al., 1978).

The active form of folate is pteroylglutamic acid (a substituted ring linked to p-aminobenzoic acid linked to glutamic acid by a peptide bond). It is reduced at the pteridine ring position 5-,6-,7- and 8- to form tetrahydrofolate (figure-5). Methyl substitution occurs at the pteridine ring position to form N^5 –methyl-tetrahydrofolate,

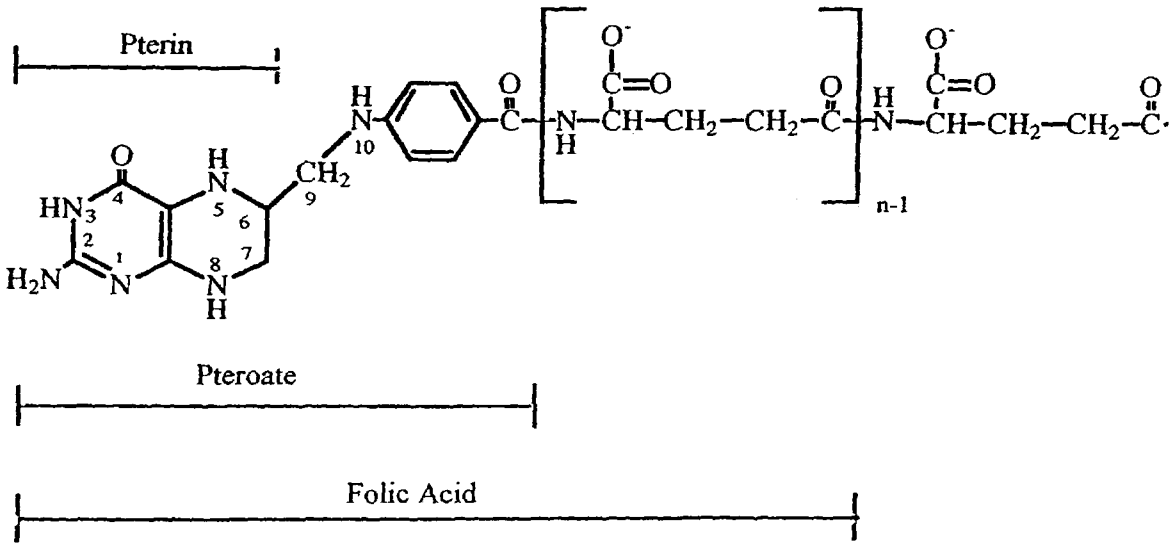


Figure 5. Biochemical structure of folate.

which is the plasma form of the vitamin. Pteroylpolyglutamate, which is the pteroylglutamate with the addition of two to seven glutamates in series, is the main dietary and intracellular form of folate (Shane and Stokstad, 1985).

The pteroylpolyglutamate has to be hydrolysed before it can be actively absorbed into erythrocytes. There are two folate hydrolases in humans, both requiring zinc, in the intestinal mucosa located in the brush-border membrane and intracellular lysosomes. Clinical studies have shown that the brush-border hydrolase of the proximal jejunum is the site where folate is digested (Halsted, 1990). The brush border 'conjugase' removes the polyglutamate chain. The monoglutamate form is presented to a specific folate receptor on mucosal cells. Over half of natural folates are in the form of 5-methyltetrahydrofolate and the rest are converted to this form in transit through the mucosal cells or in the first pass through the liver (Scott, 2000). The liver is the main site for folate storage and transmethylation with production of tetrahydrofolate and methionine. Some pteroylglutamate leaves the liver and circulates in plasma and bile. Folate is excreted daily in stool and urine, and has a biological half-life of one hundred days (Krumdieck et al., 1978).

Pteroylglutamate is taken up by bone marrow, utilised and circulated in erythrocytes as pteroylglutamate. Folate is incorporated into maturing erythrocytes. Reticulocytes are released from bone marrow into blood and circulate for one or two days, during which time they loose some of their folate. The folate stays in red blood cell throughout the cell's life of between 100- 200 days (Berlin et al., 1959). Since red blood cells are continually being produced, red blood cell folate could be a reliable indicator of folate intake over time.

Cytosolic folates are mainly pteroylglutamates, whereas plasma and urinary folates are pteroylmonoglutamate (Shane, 1990). There appears to be considerable variation in the amount and nature of folate-binding proteins in serum (Ratnam and Freisheim, 1990). Most of the cytosolic folate-binding proteins are folate-dependant enzymes (Ratnam and Freisheim, 1990). Pteroylglutamate is the folate form taken up by tissues, and methylates homocysteine, before it is polyglutamated and retained by the cells. This implies that cellular concentration of methionine affects cellular concentration of folate. Yet, if folic acid is taken, DNA synthesis and cell division occur which uses up the limited supply of methionine (protein synthesis), thereby reducing the methylation reactions even more. A lack of methylation impairs the myelination of nerves and can cause sub-acute combined degeneration of the spinal cord (Scott and Weir, 1981). For many one-carbon metabolism enzymes, the glutamyl chain length of folate affects its affinity. The one carbon moiety from N^5N^{10}-methylenetetrahydrofolate has several roles: the methylene group can be transferred to deoxiuridylate to synthesise thymidine; the methylene group could be reduced to a methyl group to form pteroylglutamate or the methylene group could be oxidised to a formyl group. Since N^5N^{10}-methylenetetrahydrofolate polyglutamate is essential for the synthesis of deoxythymidine monophosphate (V) from deoxyuracil monophosphate (V) and new cell formation, any folate deficiency will be expressed in cells of a high turnover.

In a case study, conducted on himself, Herbert (1962), showed that a folate deficient diet produced a decrease in serum folate after three weeks, tissue depletion by seven weeks, decreased folate in erythrocytes by seventeen weeks, and megaloblastic changes in the bone marrow and anaemia by twenty weeks.

Jacob et al. (1994) found that in ten healthy men, the plasma homocysteine levels rose during folate depletion. In fact, the response of homocysteine to changes in folate intake varied amongst individuals from very strong to absent. This illustrated people's genetic differences, which is their difference in folate-binding proteins in the enterocyte membranes, serum and their tissues.

Folate deficiency can occur because of decreased intake, decreased absorption, increased requirement or increased metabolism

2.8.2.2 Vitamin B_{12}

Vitamin B_{12} is a water-soluble vitamin found in meat, liver and dairy products. It is absorbed in the terminal ileum after it is bound to intrinsic factor found in gastric mucosa. Diseases of the stomach and those involving the terminal ileum can lead to deficiency states of this vitamin.

The structure of vitamin B_{12} (figure-6) is composed of a central cobalt unit and an R-group. The principal R-groups are 5'-deoxyadenosyl (5'deoxyadenosylcobal mine), - CH_3 (methylcobalmine) and a –OH (hydroxocobalmine). Methylcobalmine is the vitamin B_{12} coenzyme for methionine synthetase (homocysteine to methionine), whereas 5'-deoxyadenosylcobalmin is the coenzyme for the mitochondrial methylmalonyl-CoA mutase (methylmalonyl CoA to succinyl-CoA).

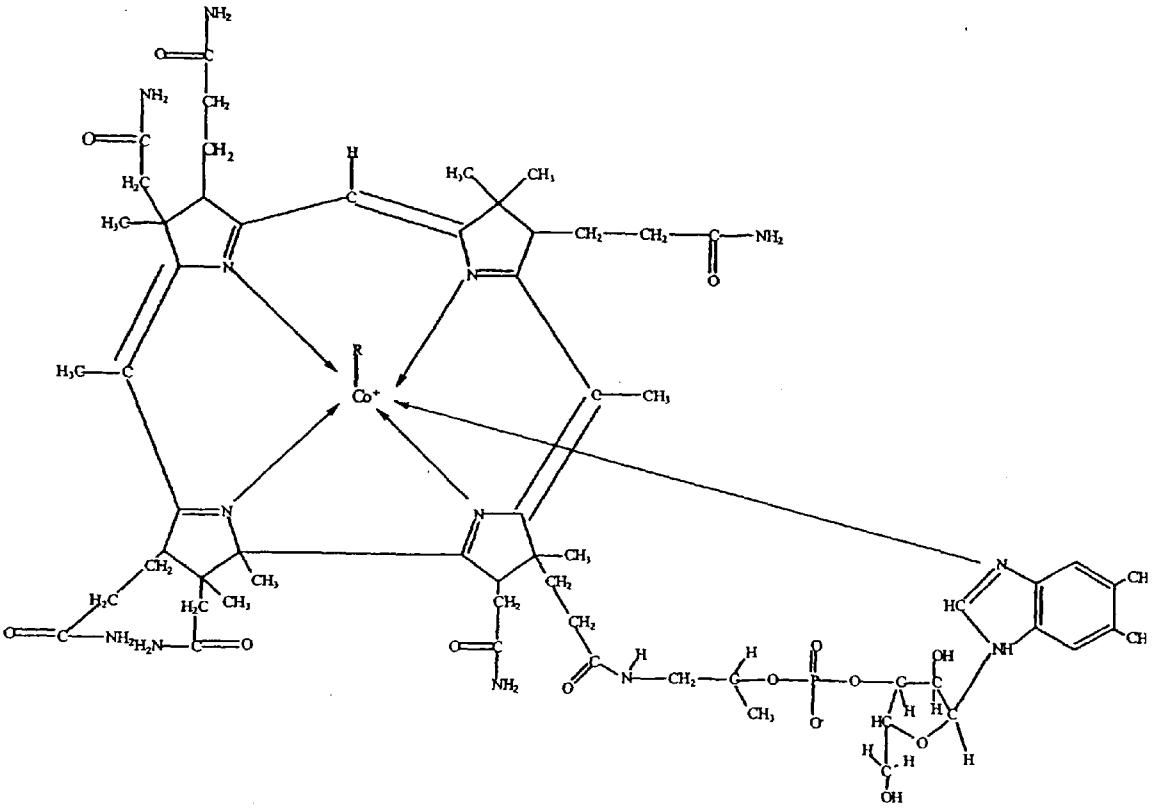


Figure 6. Biochemical structure of Vitamin B_{12}.

Dietary vitamin B_{12} is present in proteins in the food, and is liberated by the action of gastric enzyme pepsin. Vitamin B_{12} thus released binds to R proteins, which are secreted in saliva, gastric and bile fluids. The alkaline environment of the duodenum inhibits pepsin and assists in activation of pancreatic proteases, in particular trypsin, which is involved in liberation of vitamin B_{12} from the R factor. Vitamin B_{12} then binds to intrinsic factor, a glycoprotein secreted by parietal cells of the stomach (Grasbeck et al., 1966). The brush-border membrane of terminal ileum enterocytes contain a specific receptor for the vitamin B_{12} – intrinsic factor and calcium complex. Within enterocytes it is separated and transferred at the basolateral membrane to transcobalamine II and then transferred to the liver (Chanarin et al., 1978; Rothenberg et al., 1978). The transcobalamine II- B_{12} complex is taken up into tissues by specific receptors (Nexo and Hollenberg, 1980). The vitamin is excreted in bile and reabsorbed in the ileum. The body pool of vitamin B_{12} is 2-3mgs, of which 80% is in the liver and daily excretion is 1.2 to 1.3 μgm (Hall, 1964).

2.8.3 Clinical Implications

It is known that enzymatic reactions involving folic acid and vitamin B_{12} help in the formation of methionine by remethylation of excess homocysteine, thus preventing hyperhomocysteinemia and, therefore its associated clinical effects. Thus both B_{12} and folic acid regulate metabolic pathways catalysed by the enzyme methyltetrahydrofolate reductase (MTHFR). Vitamin B_6 helps in the breakdown of homocysteine into cysteine in a trans-sulphuration reaction thereby preventing excess plasma homocysteine and maintaining normal homocysteine levels.

A deficiency of folic acid is associated with neural tube defects and megaloblastic anaemia and hyperhomocysteinemia associated cardiovascular disease. Deficiency of folic acid not uncommonly occurs in alcoholics, the elderly, in those suffering with coeliac disease or tropical sprue and in epileptics taking phenytoin preparations. Multiple pregnancies, lactation, haemolytic anaemia and leukaemia can all lead to deficient folate state (Krumdieck et al., 1978; Halsted, 1990; Scott and Weir, 1981; Surtees et al., 1993).

People who are at risk of developing vitamin B_{12} deficiency are those suffering from gastrointestinal disorders, autoimmune disorders that affects parietal cells, type I diabetes mellitus, thyroid disorders, and those receiving long-term therapy with gastric acid inhibitors (Nilsson-Ehle, 1998). Deficiency of vitamin B_{12} can lead to pernicious anaemia and reduced leukocyte count. However, there is no evidence of AAA or PVD in patients with pernicious anaemia or those having terminal ileal resections. Vitamin B_{12} deficiency is also associated with neuropsychiatric disorders (Lindenbaum et al., 1988)- parasthesia, weakness, ataxia, dementia and psychosis, and subacute combined degeneration of spinal cord (Surtees et al., 1993). Patients with vitamin B_{12} deficiency have elevated plasma homocysteine and cardiovascular diseases. Malinow et al. (1996) in their review of 38 studies, found an inverse correlation between plasma homocysteine, and serum folic acid, vitamin B_{12} and vitamin B_6.

The European Concerted Action Project study (Graham et al., 1997) studied the strength of the relationship and the interaction of plasma homocysteine with other risk factors along with serum vitamins- namely folic acid, vitamin B_{12} and vitamin B_6. It

showed the patients using vitamin preparations experienced protection from vascular disease with a relative risk of 0.38 (95% CI 0.2 – 0.7), compared with non-users of vitamin supplements. It was also found that red blood cell folate concentration below the lowest tenth percentile (<513 ηg/ml) and vitamin B_6 below the twentieth percentile (<23.3 ηg/ml) for control subjects were associated with increased risk of cardiovascular disease. This was independent of conventional risk factors.

A meta-analysis of trials by Homocysteine Lowering Trialist's Collaboration (1998), of serum folic acid, vitamin B_{12} and vitamin B_6 upon total plasma homocysteine concentration, concluded that supplementation with folate reduces plasma total homocysteine concentrations, which was particularly noticeable in the highest quintile of homocysteine (18.5 μ mol/L) and the lowest quintile of serum folate (<6.9 (ηg/ml). Malinow et al. (1998) found that daily intake of fortified cereals containing about 500 μg or more of folic acid in combination with the recommended daily allowance (RDA) of other vitamins can reduce plasma homocysteine levels by 14%.

Boushey et al. (1995) calculated that an increase in folic acid intake of 200 μg/day reduces homocysteine levels by approximately 4 μg/l. Studies previously have shown a fall in homocysteine levels following administration of 650 – 10000 μg of folic acid (Dudman et al., 1993; Brattstrom et al., 1984; Brattstrom et al., 1988). A folic acid dose of 650μg reduces homocysteine levels by 42% (P<0.001) (Ubbink et al., 1994). Other authors have shown that folic acid intake between 200 to 15,000 μg/day can lower homocysteine without producing toxicity (Brattstrom, 1996; Guttormsen et al., 1996). It has been further estimated that a 25% reduction of homocysteine can be obtained with 500 to 5,700 μg of folic acid, and if 1 mg/day of vitamin B_{12} were

added a further 7% reduction of homocysteine was achieved (Homocysteine Lowering Trialists' Collaboration 1998). Supplemental vitamin B_{12} has been effective in almost 70% of cases, in normalising elevated homocysteine in cases with overt B_{12} deficiency (Lindenbaum et al., 1988). In a study using placebo as control, a combination of 0.65 mg/day of folic acid, 10 mg/day of vitamin B_6 and 0.4 mg/day of B_{12} were effective in normalising moderate hyperhomocysteinemia (Ubbink et al., 1995). However, there is no additional lowering of homocysteine after 6 weeks of supplemental intake of 1000 μg of folic acid, 0.4 mg of cobalmine and 12.2 mg of pyridoxine if twice these amounts of vitamins are given (Ubbink et al., 1993). Dudman et al. (1993) found that folic acid is the more essential amongst the vitamins in reducing fasting hyperhomocysteinemia. Cobalmine is also effective in reducing homocysteine levels in cases with overt B_{12} deficiency (Brattstrom et al., 1990).

Pyridoxine intake is effective in reducing elevated homocysteine levels following post-methionine intake but not fasting homocysteine levels (Brattstrom and Lindgren, 1992; Brattstrom et al., 1990). Other vitamins such as riboflavin in daily intakes of 0.6 mg can bring about a modest reduction in plasma homocysteine (Shimakawa et al., 1997)

In summary, the evidence strongly identifies an inverse relationship between vitamins such as folic acid, vitamin B_{12}, vitamin B_6 and plasma homocysteine. There is little evidence to date that has investigated the relationship between aneurysms, homocysteine and vitamins. It is the intention of this investigation to address this relationship.

Chapter 3

Aims of the study

3.0 Aims of the study and Null hypothesis

This thesis consisted of two studies:

Study 1. The aim of this study was to investigate the relationship between elevated plasma homocysteine levels (>15 μmol/L), AAA, and serum vitamin B_{12} and folic acid.

Study 2. The aim of this study was to investigate possible relationship between elevated plasma homocysteine levels (>15 μmol/L), PVD, and serum vitamin B_{12} and folic acid.

3.1 Null Hypothesis (H_0)

Null Hypothesis (1) There are no differences in total plasma homocysteine, vitamin B_{12} and folic acid between patients with AAA, PVD, and healthy control.

Null Hypothesis (2) There are no relationships between total plasma homocysteine, vitamin B_{12} and folic acid.

Chapter 4

Materials and Methods.

4.0 Materials and Methods.

4.1 Sample size

Anderson et al. (1992) found the mean plasma total homocysteine concentration in healthy subjects was 9.7 ± 2.4 μmol/l standard deviation (standard deviation 24.7%). Rossi et al. (1999) found that the biological coefficient of variation was 8.3% and the analytical coefficient of variation was 3.1% for 20 subjects. Similar figures were found by Kuo et al. (1997). The critical difference was 24.6%, therefore the standardized difference was 24.6/24.7 = 1.00. The number of subjects for the power of the test to be 0.9 with a significance level of <0.05 using the nomogram was 30 (Altman, 1980; Altman, 1991).

4.2 Number of subjects and the critical variance

Altman, (1980) suggested that the smallest sample size for comparing two independent groups when the variable is continuous, could be calculated, assuming that the variable has a normal distribution. The total sample size is N, the power of the test will be 0.90 (90%) with a significance of 0.05 (5%). The standardized difference (SD) is the ratio of the clinically relevant difference to the standard deviation of the variable for the study groups.

$$SD = \frac{\delta}{s} = K \times \frac{\text{square root of } (CV_a^2 + CV_w^2)}{s}$$

δ = critical difference

K = 2.77 for P< 0.05

CV_a = coefficient of analytical variation

CV_w = coefficient of within subject variation(biological variation).

s = standard deviation of the changes expected (SD/ X)

$CV_a = SD_a / X$

$CV_w = SD_w / X$

The study sample size (N) was obtained from the nomogram (Altman 1980). A ruler was placed beside the calculated standardised difference value and the hypothetical power of the test value. The size of the study was read off when the edge of the ruler passed through the 0.05 significance line. The power of the test suggested a sample size of 30 patients in each group.

Thirty-six patients with PVD and 36 control subjects and 38 patients with AAA were recruited for the study. More than 30 subjects in each groups were selected because more than expected number of subjects turned up on the final day of sample collection, and presented the opportunity to collect samples on additional subjects in case of any eventuality with the 30 samples in each group.

4.3 Ethical Approval, Informed Consent, Patient Information Sheet and Patients Characteristic (Risk factor) sheet

Ethical approval was obtained from the Bro Taff Local Research Ethics Committee. The subjects were completely informed about the protocol and study. They were given a patient information sheet and informed consent was obtained.

Another sheet was used to record the characteristic or risk factors for individual patients.

Appendix - 1. Patient Information Sheet

Appendix - 2. Informed Consent

Appendix - 3. Patient Characteristic (Risk Factor) Questionnaire Sheet

4.4 Study design

This was a case-control study.

4.5 Subject selection:

Patients and controls were recruited from the database for ultrasound scan (USS) screening programme for AAA. Those with an abdominal aortic diameter of > 3 cm were randomly selected for abdominal aortic aneurysm AAA group. Those with a normal scan and no symptoms of PVD and ABPI of 1 or greater were selected for control group. Patients with clinical symptoms of peripheral vascular disease and confirmed ABPI of 0.8 or less and a normal USS of the abdomen were randomly selected for peripheral vascular disease group. Thus there were three groups: 1. AAA. 2. PVD. and 3. Control.

Group 1- (AAA group) consisted of patients with abdominal aortic aneurysm (AP diameter of 3cm or more) diagnosed on ultrasound scan (using Gray-scale B-mode images) of the abdomen.

Group 2- (Control group) consisted of individuals free from any known AAA which was excluded using an ultrasound abdominal scan, and free of obvious PVD, assessed clinically and on the basis of ankle brachial ratio of 1 or greater. These individuals were randomly selected from the open access ultrasound scan screening

programme.

Group3 – (PVD group) consisted of patients with PVD (history of intermittent claudication or rest pain and confirmed ABPI of 0.8 or less). These patients also underwent USS of the abdomen to exclude any AAA. Thus this group of patients had PVD but no clinical or USS evidence of AAA.

4.6 Exclusion criteria

Patients, who had not fasted for 8 hours prior to blood sample withdrawal, those who were taking any vitamin supplement, or whose blood sample could not be centrifuged for separation of serum within one hour of collection, were excluded from the study.

4.7 Collection of samples

The researcher carried out the blood collection and storage. Approximately 4 ml of venous blood were taken from the antecubital vein into a vacutainer tube containing EDTA (Beckton-Dickinson, Newbury, Berkshire). The sample was immediately transferred to an ice bag and taken to the biochemistry laboratory at the Royal Glamorgan Hospital. Within 60 minutes of collection, blood samples were placed in a separator and centrifuged at 3,000 revolutions per min for 5 minutes, to separate the supernatant serum from the cells. This serum was stored at –80°C prior to the analysis of plasma homocysteine concentration.

For vitamin analysis, serum was obtained from another EDTA tube using the same centrifugation process. This serum was used to measure Vitamin B_{12} and folic acid.

The analysis of the samples were undertaken by biochemists having expertise in this field.

4.8 Biochemical Analysis

4.8.1 Homocysteine

Fasting homocysteine serum levels were analysed using a 'fluorescence polarisation immunoassay (FPIA) technique for the quantitative measurement of total homocysteine' (IMx system, Axis biochemicals, ASA Ulvenvein, Norway). Plasma homocysteine levels > 15 μmol/L were taken as abnormally elevated (Ueland et al., 1993).

4.8.2 Vitamins

Vitamins B_{12} and folic acid were measured with an automated immunoassay analyser, using a chemiluminescence detection technique (Bayer ACS 180, Newbury, Berkshire). The normal range of vitamin B_{12} was 160 to 350 pg/L and that of folic acid was 3.5 to 15 (ηg/ml).

In addition, urine specific gravity and haematocrit were ascertained to facilitate correction for concentration factor difference between the two groups that could have been induced due to overnight fasting.

4.9 Ultrasound scan (USS) of the abdomen

Ultrasound was performed using a Diasonic DRF 100 real-time scanner (Diasonic Sonoton Ltd, Bedford, U.K.), with a 3.5 MHz curved array transducer (Aloka SSD-2000, Japan).

All USS were performed by a single vascular technician who had formal training in such procedure and had experience of scanning several hundred patients. Patients' consent was obtained and the procedure of the USS of the abdomen explained, stressing that it is a simple, safe, painless and non-invasive procedure, which normally takes about 10 minutes. The patients lay supine on the examination bed with the abdomen exposed from the level of xiphisternum to the pubis. Lubricant jelly was used to facilitate movement of the USS hand held probe on to the abdomen. Gray-scale B-mode images of the abdominal aorta was obtained. The normal diameter of the abdominal aorta was taken as 2 cm or less and an aorta was defined to be aneurysmal when the anterioposterior (AP) diameter was considered to be 3cm or greater (Johnston et al., 1991; Lindholt et al., 1997). Printed records of all the aneurysms were obtained. All those with a positive scan were entered into the USS surveillance programme to be subsequently followed-up according to the protocol. Those who agreed to participate in the study were entered into the AAA group. Those with a normal scan were reassured, their details recorded, and 36 subjects amongst these who agreed to participate in the study were entered into the control group after testing for absence of peripheral vascular status i.e. absence of clinical symptoms of PVD and ankle brachial pressure index (ABPI) of 1 or greater. The subjects were selected randomly.

4.10 Ankle brachial pressure index (ABPI)

ABPI was measured using a hand-held Mini Doppler (model no. D 900, Huntleigh, Cardiff, U.K.) and a standard sphygmomanometer.

In peripheral arterial disease the signs and symptoms in the lower limbs are due to the absolute or relative decrease in the blood pressure or blood flow due to obstructions in the proximal aorta or its branches that take care of the peripheral circulation. Blood pressure was measured at the brachial artery (brachial pressure) medial to the brachialis tendon at the elbow, using a pocket doppler with a hand held 7 to 10 MHz probe and standard blood pressure cuff measuring 12 cm in width, wrapped around the arm above the elbow. Blood pressure at the ankle (ankle pressure) was measured using the same cuff around the calf and the doppler targeting the posterior tibial artery below the medial malleolus. The anterior tibial artery was not used because it is less reliable and more easily affected by calcification in conditions such as diabetes. The ABPI is calculated by dividing the systolic pressure at the posterior tibial artery by the systolic pressure at the brachial artery. The normal ABPI is 1 or greater (Carter, 1968; Yao, 1969; Yao, 1970). Those between 0.9 to <1 are considered equivocal, whereas those <0.9 are considered to be indicative of PVD. Those with ABPI 0.8 or less were selected randomly into the PVD group. These groups of patients had been selected from the out-patients clinic where they were referred for specialist vascular assessment.

4.11 Summary and explanation of test

Homocysteine (Hcy) is a thiol-containing amino acid produced by the intracellular demethylation of methionine. Homocysteine is exported into plasma

where it circulates, mostly in its oxidized form, bound to plasma proteins as protein-Hcy mixed disulphide with albumin (Malinow, 1994). Smaller amounts of reduced homocysteine and the disulphide homocysteine (Hcy-SS-Hcy) are present. Total homocysteine (THcy) represents the sum of all Hcy species found in plasma or serum (free plus protein bound).

Homocysteine is metabolised to either cysteine or methionine. A major part of homocysteine is remethylated to methionine, mainly by the folate and cobalmine dependent enzymes, methylene tetrahydofolate reductase (MTHFR), homocysteine methionine methyl transferase (HMMT) and methionine synthetase. In the vitamin B_6 dependent trans-sulphuration pathway, homocysteine is irreversibly catalysed to cysteine with the help of enzyme of cystathione beta synthetase. Homocysteine accumulates and is excreted into the blood when these reactions are impaired (Ueland and Refsum, 1989).

Severely elevated concentrations of total homocysteine are found in subjects with homocysteinuria, a rare genetic disorder of the enzymes involved in the metabolism of homocysteine. These patients exhibit mental retardation, early arteriosclerosis and arterial and venous thromboembolism (Mudd et al., 1995). Other less severe genetic defects, which lead to moderately elevated levels of total homocysteine, are also found. Epidemiological research has shown that different patient groups might have elevated total homocysteine levels in blood. A meta-analysis of 27 studies including more than 4,000 patients estimated that a 5 μmol/L increase in total homocysteine was associated with an odds ratio of 1.7 for coronary artery disease, 1.5 for cerebrovascular disease or the same increase in risk as for a 0.5 μmol/L increase in cholesterol (Boushey et al., 1995).

Patients with chronic renal disease experience an excess morbidity and mortality due to arteriosclerotic cerebrovascular disease (CVD). Elevated concentration of total homocysteine is a frequently observed finding in the blood of these patients. Although they may lack some of the vitamins involved in the metabolism of homocysteine, the increased levels of total homocysteine are mainly due to impaired removal of homocysteine from the blood by the kidney (Bostom and Lathrop, 1997). Drugs such as methotrexate, carbamezapine, phenytoin may elevate levels of homocysteine (Ueland et al., 1993).

4.12 Biological principles of the procedure

The homocysteine assay is based on Fluorescence Polarization Immunoassay (FPIA) technology.

Bound homocysteine (oxidized form) is reduced to free form. Free homocysteine is enzymatically converted to S-adenosyl-L-homocysteine (SAH) as outlined below.

4.12.1 Reduction:

Homocysteine and mixed disulphide and protein-bound forms of Hcy in the sample are reduced to form free Hcy by the use of dithiothreitol (DTT).

Hcy-SS-Hcy(Homocysteine)

R_1 –SS-Hcy (R_1= thiol residue) $\xrightarrow{\text{DTT}}$ Hcy

Protein-SS-Hcy

4.12.2 Enzymatic Conversion:

Total free Hcy is converted to S-adenosyl-L-homocysteine (SAH) by the use of SAH hydrolase and excess adenosine.

Hcy + Adenosine $\xrightarrow{\text{SAH-hydrolase}}$ SAH

The IMx homocysteine reagents and sample are added to the FPIA sample cartridge and cuvette in the following sequence:

- The probe/electrode assembly delivers the sample, pre-treatment solution, enzyme, and FPIA #1 diluent buffer to the predilution well of the sample cartridge.
- The pre-treatment solution reduces homocysteine and mixed disulphide and protein bound forms of Hcy present in plasma or serum into one chemical form, homocysteine.
- The enzyme converts homocysteine to S-adenosyl-L-homocysteine (SAH)
- An aliquot of the pre-dilution mixture, antibody, and FPIA #1, diluent buffer are delivered to the cuvette and a background measurement is made by the FPIA optical assembly.
- Tracer, FPIA#1 diluent buffer, and a second aliquot of the pre-dilution mixture are transferred to the cuvette.
- SAH and labelled fluorescein tracer compete for the sites on the monoclonal antibody molecule.
- The intensity of polarized fluorescent light is measured by the FPIA optical assembly.

4.12.3 Expected values

Scientific literatures suggest the normal values of total homocysteine concentration in plasma or serum of healthy adults to be between 5 and 15 μmol/L (Ueland et al 1993).

4.13 Statistics

4.13.1 Normality

The data symmetry (skewness) and kurtosis were obtained using SPSS computer software. Data were analysed using non-parametric tests.

4.13.2 Statistical analysis

A one-way analysis of variance (ANOVA) was used to test the significance of differences in plasma total homocysteine, vitamin B_{12} and folic acid between the AAA, PVD and control groups. Post-hoc comparisons of differences between mean values were performed using Scheffe's Confidence Interval test. Data are presented as mean ± standard errors of the mean (SE). Differences were considered significant at the 95% level *($P < 0.05$).* Pearson correlation was used to examine the relationship between plasma homocysteine and B_{12}, plasma homocysteine and folic acid, and plasma homocysteine and the size of AAA, significance level at $P < 0.01$. Difference in the characteristics or risk factors in the control, AAA and the PVD groups were analysed using Chi square and Fischer's exact test. All statistical tests were performed using SPSS computer software package.

Chapter 5

Study 1

Plasma Homocysteine, Vitamins and Abdominal Aortic Aneurysms.

5.1 Abstract

Background

Hyperhomocysteinemia is a recognised independent risk factor in the genesis of atherosclerotic vascular diseases. However very little is known about the relationship between homocysteine and abdominal aortic aneurysm (AAA). Vitamins, namely B_{12} and folic acid have been implicated in the regulation of plasma homocysteine levels. However, there has been no prospective study that has analysed the relationship of AAA with plasma homocysteine and serum vitamin levels.

Aims

To study the relationship between plasma homocysteine and AAA in conjunction with serum B_{12} and folic acid levels.

Method

Case control study including 38 AAA patients and 36 controls. Fasting homocysteine, B_{12} and folic acid were determined in serum separated within one hour of blood collection using a fluorescence polarisation immunoassay technique (FPIA).

Results

26 (68%) of the AAA patients had elevated levels of homocysteine compared to 2 (6%) in the case control group. The mean homocysteine level in the AAA group was 19.4 μmol/L (95% CI 17.17 – 21.65) and in the control group was 10.9 μmol/L (95% CI 9.95 – 11.88), ($P< 0.001$). Mean Vitamin B_{12} levels in the AAA and controls were 332.11 and 414.33 pg./L (SE ± 19.72) respectively ($P < 0.004$). Mean Folic acid in the AAA group was 8.02 (SE ± 0.71) and in control was 9.8 ηgm/L (SE ± 0.69)

respectively ($P > 0.05$).

Conclusion

This study confirms significantly higher levels of plasma homocysteine in AAA patients but lower levels of B_{12}. Use of supplemental vitamins that lower plasma homocysteine may modify vascular disease progression and clinical trials in this direction are warranted.

5.2 Introduction

Homocysteine biochemistry is complex, with the plasma total homocysteine (THcy) being the sum of homocysteine, homocystine, and the homocysteine – cystine mixed disulphide, free and protein bound (Fortin and Genest, 1995). It was the observation that patients presenting with homocysteinuria, an autosomal recessive metabolic disorder leading to raised plasma homocystine, also displayed premature vascular disorders that drew attention to the causal relationship between vascular disorder and homocystine (McCully, 1969; Gibson, 1964; Shimke, 1965). It was subsequently shown in experimental models that intravenous infusion of homocysteine caused endothelial vascular injury and atherosclerosis (Harker, 1976). Further studies demonstrated that elevated levels of homocysteine were a risk factor in the causation of coronary artery (Wilcken and Wilcken, 1976), cerebrovascular (Brattstrom et al., 1984) and peripheral vascular diseases (Boers et al., 1985), independent of other risk factors such as age, gender, lipids, lipoproteins, cholesterol, hypertension and smoking. (Graham et al., 1997; Boushey et al., 1995; Clarke et al., 1991). There are currently very little data that have examined the possible relationship between plasma homocysteine and the development of abdominal aortic aneurysm (AAA) (Brunelli et al., 2000) and serum vitamin levels. Therefore, this study was designed to investigate possible correlation between THcy, AAA, and serum vitamin B_{12} and folic acid levels.

5.3 Materials and Methods.

5.3.1 Study design

This was a prospective study in which patients were selected into 2 groups.

Group1- (AAA group) which consisted of patients with AAA (AP diameter ≥ 3cms) diagnosed on ultrasound scan (USS) (using Gray-scale B-mode images) of the abdomen.

Group 2- (Control group) which consisted of individuals free from any known AAA (excluded using an ultrasound abdominal scan), and free of obvious peripheral vascular disease, assessed clinically and on the basis of ankle brachial ratio >0.9. These individuals were randomly selected from the open access aneurysm USS screening programme.

Please see materials and methods, chapter 4, for details of the methodology.

5.4 Results

There were a total of 74 subjects, 38 in the AAA group with a mean age of 70 years (range 53- 79) ± SE 0.92, and 36 in the control group with a mean age of 66 years (range 48- 79) ± SE 1.2.

The characteristics (disease and lifestyle) of patients in the AAA group and the control group are summarised in Table-1.

The mean homocysteine concentration for patients in the AAA group was 19.4 ± SE 1.1 μmol/L (95% CI 17.17 – 21.65) and for subjects in the control group 10.9 ± SE 0.47 μmol/L (95% CI 9.95 – 11.88) (Table-2). The difference between the two groups was highly significant ($P < 0.001$). In the AAA group the mean homocysteine levels for patients ≥ 70 years compared to those < 70 years was 19.2 ± SE 0.8 μmol/L and 19.6 ± SE 0.9 μmol/L respectively ($P > 0.05$). For the control group these values were 11.3 ± SD 0.44 vs 10.3 ± SE 0.42 μmol/L ($P > 0.05$). The distribution of homocysteine in the AAA and control groups is shown in figures 7. The mean homocysteine level in AAA ≤ 4 cm and those > 4cm were 18.8 ± SE 0.1.3 μmol/L and 20.3 ± SE 1.9 μmol/L respectively ($P > 0.05$). There was no correlation between the size of AAA and plasma homocysteine level ($r = 0.014$, $P > 0.05$) (figure 8).

There were 35 men and 3 women in the AAA group and 21 men and 15 women in the control group. The mean homocysteine for males in AAA group was 19 ± SE 0.7 μmol/L and for females was 23.4 ± SE 1.1 μmol/L ($P > 0.05$). The mean homocysteine for men and women in the control group was 11.5 ± SE 0.6 vs 10.2

± SE 0.5 μmol/L respectively ($P > 0.05$). These differences for age and sex were not significant.

The mean vitamin B_{12} in the AAA group was 332.11 ± SE 16.44 pg/ml and in the control group was 414.33 ± SE 19.72 pg/ml. The difference between the two groups was highly significant ($P < 0.004$). The mean folic acid in the AAA group was 8.02 ± SE 0.71 ηg/ml and control group was 9.8 ± SE 0.69 ηg/ml ($P > 0.05$). There was no statistically significant difference in the folic acid levels between the two groups.

The urinary specific gravity in the AAA group was 1015 ± SE 0.91 and Control group was 1018 ± SE 1.42 ($P > 0.05$). The haematocrit in the AAA group was 43.52 ± SE 0.67 and Control group was 43.82 ± SE 0.68 ($P > 0.05$). These differences were not statistically significant, indicating comparability of the 2 groups in terms of hydration status.

Table-1. Characteristics (disease and lifestyle) of patients in the AAA group and the control group

Characteristics	**AAA**	**Control**	**'P'**
Hypertension	13	12	0.955
Hyperlipidaemia	4	3	0.769
Diabetes	3	4	0.677
COAD	3	0	0.097
Cardiac disease (MI, angina)	24	9	0.0385
Stroke	5	1	0.130
Family history of AAA	4	0	0.0572
Constipation	8	2	0.0875
Cancer	3	3	0.949
Benign hypertrophy of prostate	8	6	0.690
Smoking	13	12	0.955
Alcohol (regular intake)	7	9	0.581

Table-2. Plasma homocysteine and serum vitamins– B_{12} and folic acid, in AAA and control groups. Values are mean ± SE of the mean

Mean Values	**AAA**	**Control**	**'P' value**
Homocysteine (μmol/L)	19.4 ± SE 1.1	10.9 ± SE 0.47	<0.001
Vitamin B_{12} (pg/ml)	332.11 ± SE 16.44	414.33 ± SE 19.72	< 0.004
Folic acid (ηg/ml)	8.02 ± SE 0.71	9.8 ± SE 0.69	=0.1 (ns)

Distribution of THcy in the AAA and controls

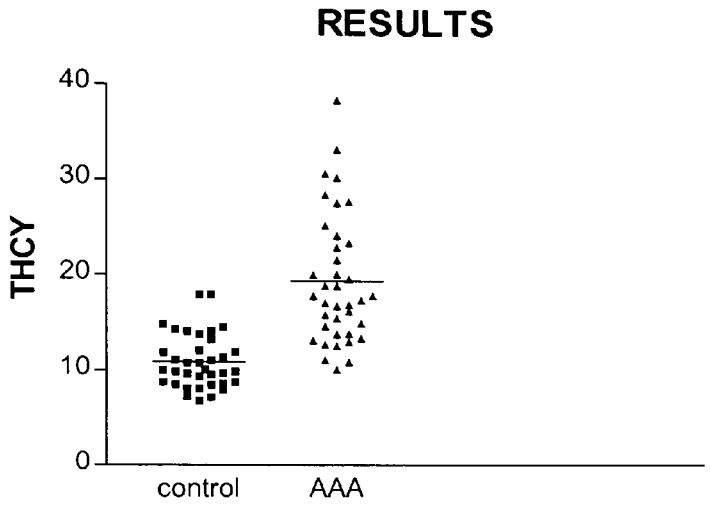


Figure 7. Plasma homocysteine (THcy) (μmol/L) in the controls, and patients with AAA. The transverse line in the scatterplot represents the median value.

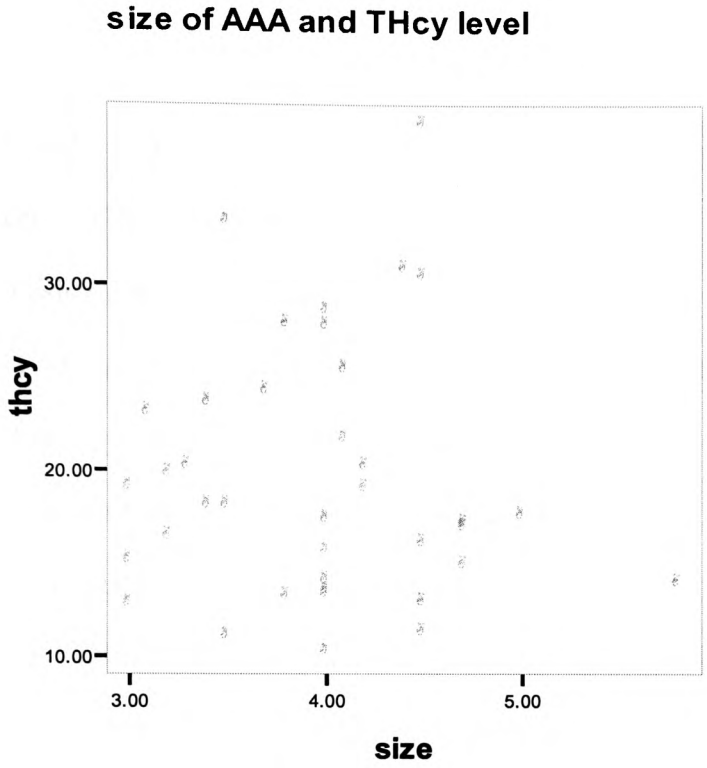


Figure 8. Plasma homocysteine (THcy) (μmol/L) on the Y axis and size of the AAA (cm) in individual patients in this group on the X axis. There is no correlation (r = 0.014, P > 0.05).

5.5 Discussion

The primary finding of this study is that plasma homocysteine levels of patients with AAA are significantly higher than those of the control group. The importance of homocysteine in coronary artery disease, cerebrovascular disease and peripheral vascular disease is well documented (Boers et al., 1985; Brattstrom et al., 1984; Wilcken and Wilcken, 1976). However, current literature on the role of homocysteine in the aetiopathogenesis of AAA is limited (Brunelli et al., 2000; Mohan et al., 1997; Colwell et al., 1991; Almgren et al., 1978). 26 of 38 (68%) patients in the AAA group had raised plasma homocysteine compared to only 2 of the 34 (6%) patients in the control group. Previous authors have found a prevalence of hyperhomocysteinemia in 40% of patients with peripheral vascular disease (Clarke et al., 1991) and 48% with AAA (Brunelli et al., 2000). Present data suggests that there may be a higher prevalence of hyperhomocysteinemia in AAA than previously believed. A 6% prevalence of hyperhomocysteinemia in the control group is very similar to 5 and 7% reported in previous studies (Ueland and Refsum, 1989; McCully, 1992). In the AAA group, there was no correlation between the size of the AAA and the levels of homocysteine. Though there is evidence that homocysteine is associated with aortic atherosclerosis (Konecky et al., 1997) and AAA (Brunelli et al., 2000), it is possible that hyperhomocysteinemia initiates the atherosclerosis but once an aneurysm is established any subsequent dilatation is more of a mechanical event in relation to the blood pressure in the AAA, and with time the AAA progressively dilates.

Four patients in the AAA group had familial history of AAA against none in the control group. This raises the question whether the familial nature of AAA is due to higher plasma homocysteine, as hyperhomocysteinemia can itself be familial. It is

well known that patients with homocysteinuria, an autosomal metabolic disorder leading to elevated plasma homocysteine, develop premature vascular disease. (McCully, 1969; Gibson et al., 1964).

Plasma homocysteine levels have been found to increase with age in some (Verhoef et al., 1999; Andersson et al., 1992) but not all previous studies (Kawashiri et al., 1999; Currie et al., 1996). This study found no such association when plasma homocysteine levels analysed in relation to age, in either the AAA or the control group. Similarly, there was no association of plasma homocysteine to sex. The homocysteine levels have been shown to be greater in men than women by most authors (Lupattelli et al., 1999; Cheng et al., 1997; Selhub et al., 1999) but some have shown no difference or marginally higher homocysteine levels in women than men (Jacobsen et al., 1989; Quiroga et al., 2001).

It is known that enzymatic reactions involving folic acid and vitamin B_{12} help in the formation of methionine by remethylation of excess homocysteine, thus preventing hyperhomocysteinemia. Vitamin B_6 assists in the breakdown of excess homocysteine into cysteine in an irreversible trans-sulphuration reaction and thus maintains normal homocysteine levels. Previous studies have examined the relationship between folic acid, B_{12} and their influence on the homocysteine levels (Chambers et al., 2000; Koehler et al., 1997; Selhub et al., 1999; Koehler et al., 1996) These studies have shown that hyperhomocysteinemia is associated with deficiencies of vitamin B_{12} and folate levels. The strongest inverse correlate for patients with coronary disease being between homocysteine and folate (Temple et al., 2000; Verhoef et al., 1996; Clarke and Armitage, 2000).

These data have shown that the mean vitamin B_{12} levels were significantly higher in the AAA group than the control group. Although the mean folic acid levels were greater in AAA in comparison to control this failed to reach statistical significance. Present data suggests that the homocysteine levels are more susceptible to changes in vitamin B_{12} as opposed to folic acid levels although, previous studies have shown low folate deficiency may be more important than vitamin B_{12} deficiency in influencing the plasma homocysteine levels and vascular diseases (Temple et al., 2000; Verhoef et al., 1996). Vitamin B_6 was not measured in the study because the Homocysteine Lowering Trialist's Collaboration, (1998), showed that supplemental doses of vitamin B_6 does not confer any additional protection unlike folic acid and vitamin B_{12}.

In conclusion, this study demonstrates a significantly higher level of plasma homocysteine but lower level of vitamin B_{12} in the AAA group in comparison to the control group. In order to confirm the relationship, long-term population studies will be required to establish whether patients at risk of AAA can be prevented from developing aneurysm by selected vitamin supplements.

Chapter 6

Study 2

Plasma Homocysteine Levels, Vitamins and Peripheral Vascular Disease

6.1 Abstract

Background

Elevated levels of plasma homocysteine is a recognised independent risk factor in the genesis of atherosclerotic diseases. Previous studies have shown association between hyperhomocysteinemia and vascular diseases. However, some authors have shown no such associations. Until large scale randomised controlled trials to study plasma homocysteinemia in patients with PVD vs control subjects are undertaken and results known some degree of controversy may remain with regards to correlation between homocysteine and peripheral vascular. Vitamins namely B_{12} and folic acid are known to be involved in the metabolism and regulation of plasma homocysteine levels.

Aims

To study the relationship between plasma homocysteine and peripheral vascular disease in conjunction with serum vitamin levels i.e. B_{12} and folic acid.

Method

Case-control study with age matched control group. Fasting homocysteine and serum vitamin levels were analysed using FPIA. Serum was separated within one hour of blood collection from antecubital venous puncture into an EDTA primed tube, from 36 PVD patients and 36 case controls.

Results

Twenty-six (72%) of the PVD patients had elevated levels of homocysteine compared with 2 (6%) in the control group. The mean homocysteine level in the PVD group was 18.4 ± SE 0.99 μmol/L (95% CI 16.32 – 20.35) and in the control group was 10.9 ± SE 0.47 μmol/L (95% CI 9.95 – 11.88), (P< 0.001). Mean Vitamin B_{12} in the PVD and control groups was 320.47 ± SE 16.81 and 414.33 ± SE 19.72 pg/L respectively (P < 0.004). Mean folic acid in the PVD was 7.84 ± SE 0.81 and in control was 9.8 ± SE 0.69 ηg/L respectively (P > 0.05).

Interestingly, there was an inverse relationship between plasma homocysteine levels and serum B_{12} (P=0.000) (r= -0.42) as well as plasma homocysteine and folic acid (P= 0.001) (r= -0.32) (Pearson correlation, significance taken at P < 0.01), when all the 110 patients (38 AAA, 38 control and 36 PVD) were taken into account.

Conclusion

This study confirms significantly higher levels of plasma homocysteine in PVD patients but lower levels of B_{12}. Use of supplemental vitamins that should lower plasma homocysteine may modify vascular disease progression and clinical trials in this direction are warranted.

6.2 Introduction

Studies have demonstrated that elevated levels of homocysteine is a risk factor in the causation of coronary artery (Wilcken and Wilcken, 1976), cerebrovascular (Brattstrom et al., 1984) and peripheral vascular diseases (Boers et al., 1985), independent of other risk factors such as age, gender, lipids, lipoproteins, cholesterol, hypertension and smoking (Graham et al., 1997; Boushey et al., 1995; Clarke et al., 1991). However, some authors (Mudd et al., 1985) have shown no such associations though they have found it difficult to attribute any obvious reason for this. Despite several studies on plasma homocysteinemia and PVD, some degree of controversy remains about the association of homocysteine and peripheral vascular diseases. Vitamins, namely B_{12} and folic acid have been implicated in the metabolism and regulation of plasma homocysteine levels.

6.3 Aim

The aim of this study was to investigate the relationship between elevated plasma homocysteine levels (>15 μmol/L), serum vitamin B_{12} and folic acid, and PVD.

6.4 Materials and Methods

6.4.1 Study design

This was a case-control study in which patients were selected into 2 groups.

Group1- which consisted of patients with PVD (history of intermittent claudication or rest pain and confirmed ankle brachial ratio of < 0.9).

Group 2- was the control group which consisted of individuals free from any obvious peripheral vascular diseases, assessed clinically and confirmed to have an ankle brachial ratio of >9.

Please see materials and methods, chapter 3, for details of the methodology.

6.5 Results

The characteristics or the risk factors of patients in the PVD group and the control group are as shown in table-3.

There was a total of 72 patients, 36 in the PVD group, mean age 65.4 ± SE 1.42 (range 45- 78), and 36 in the control group, mean age 66 ± SE 0.77 (range 48- 79). The mean total homocysteine concentration for patients in the PVD group was 18.4 ± SE 0.99 μmol/L (95% CI 17.16.3 – 20.35) and for patients in the control group 10.9 ± SE 0.47 μmol/L (95% CI 9.95 – 11.88) (table-4). The difference in the two groups was highly significant ($P < 0.001$). The plasma levels of homocysteine reached for individual patients in the PVD and the control groups are shown in figure 9.

In the PVD group the mean homocysteine levels for patients ≤ 70 years vs > 70 years was 17.63 ± SE 0.83 μmol/L and 20.8 ± SE 1.12 μmol/L respectively ($P > 0.05$). For the control group these values were 11.3 ± SE 0.49 vs 10.3 ± SE 0.44 μmol/L ($P > 0.05$).

There were 28 males and 8 females in the PVD group and 21 males and 15 females in the control group. The mean homocysteine for males in PVD group was 18.9 ± SE 0.99 μmol/L and for females was 17.7 ± SE 0.89 μmol/L ($P > 0.05$). The mean homocysteine for males vs females in the control group was 11.5 ± SE 0.48 μmol/L vs 10.2 ± SE 0.43 μmol/L ($P > 0.05$).

The urinary specific gravity in PVD group was 10.17 and that in the control was

control 10.18($P > 0.05$). The packed cell volume or the haematocrit in the PVD group was 43.5 and in the control group was 43.8 ($P > 0.05$).

Mean vitamin B_{12} in the PVD group was 320.47 ± SE 16.81 pg/ml and in the control group was 414.33 ± SE 19.72 pg/ml. The difference between the two groups was highly significant ($P < 0.004$).

Mean folic acid in the PVD group was 7.84 ± SE ηg/ml (standard error 0.81) (range 6.2 to 9.5) and Control was 9.8 ± SE ηg/ml (standard error 0.69) (range 8.4 to 11.2) ($P > 0.05$). (The normal reference range for folic acid was 3.2 to 15 ηg/ml).

Interestingly, this study found an inverse relationship between plasma homocysteine levels and serum B_{12} ($r = - 0.42$, $P=0.000$) as well as plasma homocysteine and serum folic acid ($r = - 0.32$, $P= 0.001$) (Pearson correlation, significance taken at $P < 0.01$), when all the 110 patients (38 AAA, 36 control and 36 PVD) were taken into account (Table-5). This may be explained by the fact that the lower levels of folic acid in the AAA and the PVD group in comparison to the control group, failed to reached statistical significance ($P= 0.1$) due to smaller sample size. However, in the presence of a bigger sample this difference became more apparent and did reach the statistical significance. There was no difference in the plasma homocysteine levels in the AAA (19.4 ± SE 1.1 μmol/L) and the PVD group (18.4 ± SE 1.0 μmol/L) ($P = 1$) nor were there any difference in the AAA and the PVD groups for serum vitamin B_{12} (332 ± SE 16.44 pg/L vs 320 ± SE 16.81 pg/L) and folic acid levels 8.02 ± SE 0.71 ηg/ml and 7.84 ± SE 0.81 ηg/ml ($P = 1$). However, there were striking similarity in the distribution of homocysteine levels in the AAA and PVD groups in comparison to controls (figure 10).

Table-3. Characteristics (disease and lifestyle) of patients in the PVD group and the control groups (Chi square or Fischers exact test as applicable).

Characteristics	**PVD**	**Control**	**'P'**
Hypertension	16	12	0.52
Hyperlipidaemia	5	3	0.23
Diabetes	7	4	0.18
COAD	2	0	0.26
Cardiac disease (MI, angina)	13	9	0.45
Stroke	5	1	0.10
Alcohol	9	9	1
Constipation	6	2	0.13
Cancer	2	3	0.33
Benign hypertrophy of prostate	3	6	0.18
Smoking	13	12	0.86

Table-4. Plasma homocysteine and serum vitamins– B_{12} and folic acid, in PVD and control groups. Values are mean ± SE of the mean

Mean Values	**PVD**	**Control**	**'P' value**
Homocysteine (μmol/L)	18.4 ± SE 1.4	10.9 ± SE 0.47	<0.001
Vitamin B_{12} (pg/ml)	320.47 ± SE 16.81	414.33 ± SE 19.72	< 0.004
Folic acid (ηg/ml)	7.84 ± SE 0.81	9.8 ± SE 0.69	=0.1

Distribution of THcy in the PVD and controls

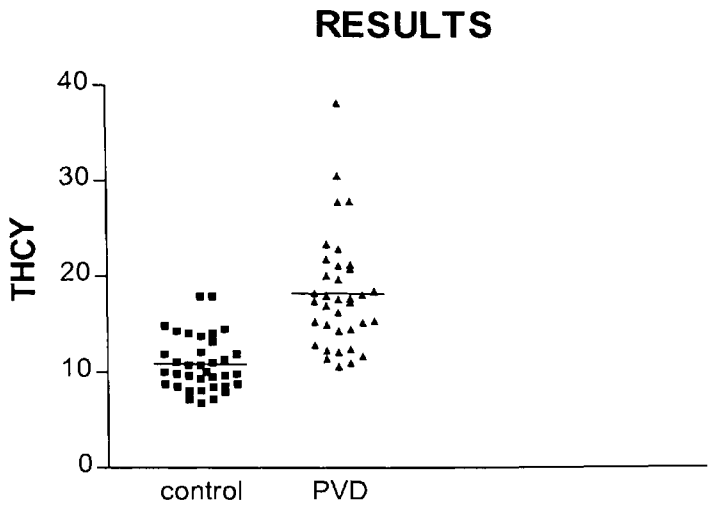


Figure 9. Plasma homocysteine (THcy) (μmol/L) in the controls and patients with PVD. The transverse line in the scatterplot represents the median value.

Distribution of THcy in the AAA, PVD and controls

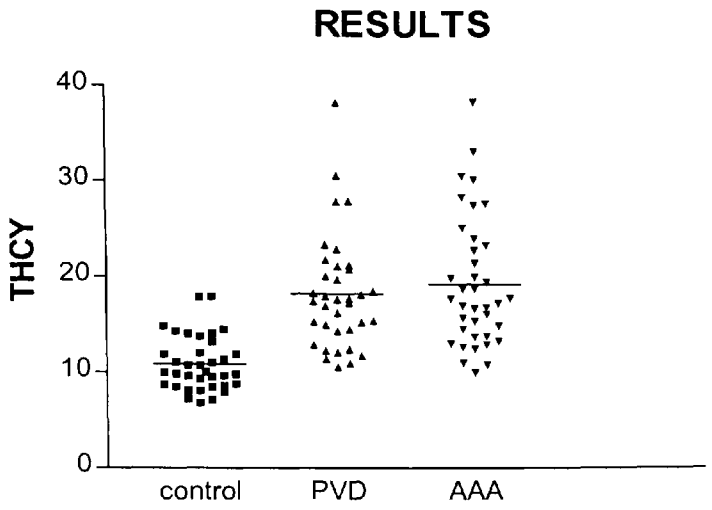


Figure 10. Plasma homocysteine (THcy) (μmol/L) in the controls, and patients with AAA and PVD. The transverse line in the scatterplot represents the median value.

Table-5. The inverse relationship between plasma homocysteine and serum vitamins-folic acid and B_{12}, using Pearson Correlation for all the 110 patients in the study.

Correlations

		THcy	B_{12} (pg/ml) 160-350	F. Acid (ηg/ml) 3.2-15
THcy	Pearson Correlation	**1.000**	**-.420(**)**	**-.326(**)**
	Sig. (2-tailed)	.	**.000**	**.001**
	N	**110**	**110**	**110**
B_{12} (pg/ml) 160-350	Pearson Correlation	**-.420(**)**	**1.000**	**.246(**)**
	Sig. (2-tailed)	**.000**	.	**.010**
	N	**110**	**110**	**110**
F. Acid (ηg/ml) 3.2-15	Pearson Correlation	**-.326(**)**	**.246(**)**	**1.000**
	Sig. (2-tailed)	**.001**	**.010**	.
	N	**110**	**110**	**110**

**** Correlation is significant at the 0.01 level (2-tailed).**

Sig. = significance

N = number of subjects

6.6 Discussion

Homocysteine and PVD

The mean level of homocysteine in-patients with PVD was 18.4 μmol/L (range 10.7 to 38.31) and that in the control was 10.9μmol/L (range 6.8 to 18). This difference between the two groups was highly significant (P <0.001). Most authors have shown a positive correlation between elevated homocysteine and PVD (Graham et al., 1997; Malinow et al., 1989; van den Berg et al., 1996) although some authors have found no correlation (Valentine et al., 1996; Mudd et al., 1981; Bunout et al., 2000). They have suggested smaller sample size, validity of their diagnosis for the vascular disease, and lack of information about the protein and vitamin intakes. Moreover, 26 of the 36 (72%) patients in the PVD group had raised plasma homocysteine compared to only 2 of the 34 (6%) patients in the control group. Previous authors have found a prevalence of hyperhomocysteinemia in 40% patients with vascular disease (Clarke et al., 1991) and 48% in patients suffering from AAA (Brunelli et al., 2000). These data suggest that there is a higher prevalence of hyperhomocysteinemia in PVD than previously reported. However, a 6% prevalence of hyperhomocysteinemia in control population is very similar to 5 to 7% reported earlier (McCully, 1969; Ueland and Refsum, 1989). The maximum concentration of plasma homocysteine in patients having PVD was 38μmol/L, which is more than twice the highest value in the control group. These data support a strong association between elevated homocysteine and PVD. Moreover, any influence of the other risk factors for PVD in this study should be regarded as minimal, since the difference in the prevalence of risk factors in the PVD and the control groups, were not statistically significant (table-3).

Homocysteine, PVD, age and sex

Plasma homocysteine levels with reference to vascular disease have been found to increase with age in some (Verhoef et al., 1999; Andersson et al., 1992) but not all the previous studies (Kawashiri et al., 1999; Currie et al., 1996). There was no such association when plasma homocysteine levels in the PVD group for patients ≤ 70 years was compared with those >70years. The same was true for patients ≤ 70 years or older vs >70 years in the control group. The mean homocysteine levels in men was 18.9 μmol/L in comparison to females 17.7 μmol/L ($P>0.05$). These data do not support the findings of previous authors who have demonstrated greater plasma homocysteine levels in men than women with vascular diseases (Lupattelli et al., 1999; Cheng et al., 1997; Selhub et al., 1999) but some authors have shown no difference or marginally higher homocysteine levels in women than men (Jacobsen et al., 1989; Quiroga et al., 2001). There were more men than women in the PVD group in this study in keeping with that previously reported (Leng et al., 2000), but the researcher cannot explain as to why the number of women is very small in comparison to the number of men, since they were selected at random.

Homocysteine, PVD and vitamins

Some authors have shown a direct relationship between plasma homocysteine and peripheral vascular disease, and an inverse relationship between homocysteine and vitamins B_{12} and folic acid, (Rassoul et al., 2000; Weiss et al., 1999) but this finding has not been consistently demonstrated (Cheng et al., 1997; Bunout et al., 2000). Previous researchers have studied vitamins- folic acid, B_{12} and B_6 intake and demonstrated an inverse relationship with plasma homocysteine levels (Chambers et

al., 2000; Koehler et al., 1997; Selhub et al., 1999; Koehler et al., 1996). It is known that enzymatic reactions involving folic acid and vitamin B_{12} helps in the formation of methionine by remethylation of excess homocysteine, thus preventing hyperhomocysteinemia. Vitamin B_6 assists in the breakdown of excess homocysteine into cysteine in an irreversible trans-sulphuration reaction and thus maintains normal homocysteine levels. This study ascertained vitamin B_{12} and folic acid because these vitamins have demonstrated a greater homocysteine lowering effect than vitamin B_6 (Clarke and Armitage, 2000; McKinley, 2000). The mean vitamin B_{12} levels of 414.33 pg/ml (SE 19.72) in the control group were significantly higher than that of 320.47 pg/ml (SE 16.81) in the PVD group ($P<0.004$). There was no statistically significant difference between the mean folic acid levels of 9.8 ηg/ml in the control group and 7.84 ηg/ml in the PVD. Previous studies have shown that serum folic acid bear stronger inverse relationship with plasma homocysteine and vascular disease than vitamin B_{12} (Temple et al., 2000; Verhoef et al., 1996). However, these data suggest that homocysteine levels are more susceptible to changes in vitamin B_{12} levels than folic acid levels in patients with PVD.

In conjunction with previous investigations these data would conclude that there is a positive correlation of PVD and plasma homocysteine with lower vitamin levels. These data are endorsed by Rassoul et al. (2000) and Weiss et al. (1999). Supplemental doses of vitamin B_{12} and folic acid may influence the homocysteine levels as a modifiable risk factor if large randomised controlled trials were to be used to study this effect. Therefore, long term follow-up of vascular disease such as PVD with supplemental vitamins that could demonstrate arrest of disease progression will be required. Such studies will eventually establish the role of hyperhomocysteinemia in the genesis of PVD and its prevention by use of prophylactic vitamins.

6.7 Recommendation for future research

These data have shown that there is a positive correlation between elevated total plasma homocysteine concentration and abdominal aortic aneurysm and peripheral vascular disease. These data have also demonstrated that there is an inverse correlation between total plasma homocysteine concentration and serum folic acid and vitamin B_{12}, and the levels of serum vitamin B_{12} are significantly lower in the abdominal aneurysm and peripheral vascular disease groups in comparison to the control group.

Selected supplemental vitamins such as folic acid and vitamin B_{12} in therapeutic doses given on a daily basis to patients with abdominal aortic aneurysm and peripheral vascular disease may possibly lower the total plasma homocysteine concentration. Patients with abdominal aortic aneurysm and peripheral vascular disease will need to be randomised into the experimental group scheduled to receive selected vitamins against control group randomised scheduled not to receive any supplemental vitamins. Serial fasting plasma homocysteine will be measured at the beginning of the study and thereafter at 1, 3, 6 and 12 monthly intervals. A significant fall in the fasting total plasma homocysteine concentration will establish the homocysteine lowering effect of folic acid and vitamin B_{12}. Whether the lower plasma homocysteine will in turn arrest or slow disease progression in patients with abdominal aortic aneurysm and peripheral vascular disease will need to be studied. This can be achieved by comparing the growth rate of aneurysm in experimental group (receiving supplemental vitamins) and control group (not receiving any supplemental vitamins) with abdominal aortic aneurysm and comparing the ABPI in experimental and control groups with peripheral vascular disease.

Appendix 1

Patient Information Sheet

It is known that an excess of or a lack of certain chemicals within the blood may damage the arteries (blood vessels) leading to a wide variety of problems such as stroke, heart attacks and gangrene. We are interested in the role these substance called homocysteine and vitamins.

We would be grateful if you could help us in our investigation by providing us with a sample of your blood, which we would use to measure the levels of these substances. All these tests could be performed during one of your routine visits to the out-patient department or on the ward if you were an in-patient.

It is hoped that the information learnt from this study will improve our knowledge of the mechanisms will lead to diseases of the arteries.

It is important for you to know that your participation in this study is entirely voluntary and you have the right to withdraw from the study at any time without it affecting your current or future care. Also, the results of your tests will be kept confidential.

If you have any further questions please do not hesitate to ask us.

Thankyou for your co-operation.

Mr. M.H.Lewis
Consultant Vascular Surgeon

Mr. A.A.Warsi
Surgical Research Fellow

Royal Glamorgan Hospital
S. Wales

Appendix 2

Informed Consent

I ..... Date of birth.....

Address.....

do hereby, consent to give venous blood, from my arm (right or left), approximately 10 mls in amount for measurement of homocysteine and vitamins for the purpose of research. I confirm that the procedure has been explained to me.

However, I am aware that I have the right to refuse, and if I choose to do so, it would not affect my right to be treated as a patient in any way.

Patient's signature

Appendix 3

Patient Characteristics or risk factors

Name

Date of Birth

Sex

Address

Size of Abdominal aorta (cms)

Date of Ultrasound Scan

Other aneurysms

Operated Yes [ ] No [ ]

Hypertension	[ ]	Hyperlipidemia	[ ]	Diabetes	[ ]
COAD	[ ]	Cardiac Disease	[ ]	Angina	[ ]
Stroke	[ ]	Family history	[ ]	Constipation	[ ]
History of Cancer	[ ]	Benign hypertrophy of Prostrate			[ ]
Smoking	[ ]	Collagen Disease	[ ]	Alcohol	[ ]
PVD	[ ]	Others	[ ]		

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